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Jordan Journal of Pharmaceutical Sciences

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INTRODUCTION

The Jordan Journal of Pharmaceutical Sciences (**JJPS**) is a peer-reviewed Journal, which publishes original research work that contributes significantly to further the scientific knowledge in pharmaceutical sciences' fields including pharmaceutical/medicinal chemistry, drug design and microbiology, biotechnology and industrial pharmacy, instrumental analysis, phytochemistry, biopharmaceutics and Pharmacokinetics, clinical pharmacy and pharmaceutical care, pharmacogenomics, bioinformatics, and also **JJPS** is welcoming submissions in pharmaceutical business domain such as pharmacoeconomics, pharmaceutical marketing, and management. Intellectual property rights for pharmaceuticals, regulations and legislations are also interesting topics welcomed from our colleagues in Schools of Law.

On a current topic in Pharmaceutical Sciences are also considered for publication by the Journal. **JJPS** is indexed in SCOPUS (Q3). It's a journal that publishes 4 issues per year since 2021 in (**March, June, September, December**). The Editorial Team wishes to thank all colleagues who have submitted their work to JJPS). If you have any comments or constructive criticism, please do not hesitate to contact us at jjps@ju.edu.jo. We hope that your comments will help us to constantly develop **JJPS** as it would be appealing to all our readers.

Prof Ibrahim Alabbadi
Editor-in-Chief
School of Pharmacy- The University of Jordan
Amman 11942- Jordan

Volume 18, 2025

Letter from the Editor-in-Chief

Life is about demand and supply. While this may sound like a business-related phrase, it is embedded in every stage of human life. The foundation of this concept is **value**—no one will buy something they perceive as worthless. However, value is subjective and depends on the buyer's perspective. For example, what is valuable to Hanan may not be valuable to Najah, even though they share the same environment and culture. The key factor here is **perception**—a term that highlights how people assess value based on their personal needs rather than through an objective and accurate evaluation. This subjectivity is also evident in research articles and submissions to the *Jordan Journal of Pharmaceutical Sciences (JJPS)*. Sometimes, two referees provide widely differing opinions on the same paper, making the decision process extremely challenging, even with a third opinion. As a result, some researchers feel discouraged when *JJPS* declines their submission. However, such decisions are never taken lightly. We strive to be as scientific, transparent, and logical as possible in our evaluations.

I am sharing this brief introduction to emphasize that all editorial board members, including myself, always strive to be as objective as possible when making decisions about accepting or rejecting submitted articles. As I write my final introduction for this year's volume of *JJPS*, I encourage all researchers to accept and understand both the outcomes that bring them joy and those that may disappoint them. After six years of continuous dedication and teamwork with my colleagues, this September I am stepping down from my position, hopeful that we will achieve a *SCOPUS Q2* ranking this year.

Over the past five years, we have made significant progress: increasing the number of issues per year from three to four, expanding the number of articles per issue from 5 to 20, and raising the total number of published articles from 15 per year to 80 in 2024, providing researchers with greater flexibility to have their valuable work published sooner. The average waiting time from submission to decision has been drastically reduced, dropping from 28 weeks to just 2.78 weeks in 2024. We have also broadened the journal's scope, ensuring a balanced and logical distribution of research across various pharmaceutical fields, including but not limited to medicinal chemistry and instrumental analysis, pharmacognosy and phytochemistry, pharmaceutics and industrial pharmacy, pharmacokinetics and pharmacodynamics, clinical pharmacy and pharmaceutical care, as well as pharmaceutical business, including pharmacoeconomics and pharmaceutical marketing. I am grateful for this journey and for the opportunity to contribute to the growth and success of *JJPS*, and I extend my sincere thanks to my colleagues and all researchers who have been part of this endeavor.

Submissions have increased dramatically, rising more than fivefold (e.g., 446 submissions in 2024) with an acceptance rate of 28.7% in the same year. Furthermore, we have received a wider diversification of submissions from countries including the USA, Canada, Australia, Europe, Iran, India, Pakistan, Bangladesh, Malaysia, Indonesia, Vietnam, Singapore, Morocco, Algeria, Tunisia, Egypt, Libya, Saudi Arabia and Gulf countries, Yemen, Lebanon, Iraq, Syria, and Jordan, submitted by researchers from both governmental and private universities, as well as scientific research institutes.

As a final note, I would like to express my sincere gratitude to my colleagues—the editorial board members, advisory board members, our dedicated editorial secretary, and the team responsible for English language editing and production. I wish them all the best, along with *JJPS*, for even greater achievements in the years to come.

Best regards

Prof Ibrahim Alabbadi
Editor-in-Chief

CONTENTS

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Investigating Factors Impacting Hospitalization Duration in COVID-19 Patients: A Retrospective Case-Control Study in Jordan

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ABSTRACT

Objective: Coronavirus disease 2019 (COVID-19) is a severe acute respiratory illness of the upper respiratory tract. Evaluating the variables that influence the period of hospitalization and complications in COVID-19 patients can help effectively and safely decrease undesirable events. Therefore, the present study aimed to evaluate parameters influencing the length of hospital stay (LOS) in COVID-19 patients.

Patients and Methods: A retrospective case-control study involving hospitalized patients due to COVID-19. Demographic information, clinical treatment, and laboratory data were obtained from their medical records. Laboratory assessments included C-reactive protein (CRP), D-Dimer, lactate dehydrogenase (LDH), ferritin, urea, creatinine, estimated glomerular filtration rate (eGFR), uric acid, and complete blood count. Statistical analyses involved bivariate and multivariate logistic regressions to identify predictors of prolonged LOS.

Results: The median LOS for 83 patients was 7 days (IQR = 6–8). The median age of the patients was 58 years. Approximately half the patients were in the high-risk group (44.6%). The multivariate logistic regression analysis revealed that high neutrophil count (AOR = 1.144 95% CI 1.059–1.236, p = 0.001) increased the odds of prolonged LOS. In contrast, high creatinine levels decreased the odds of prolonged LOS (AOR 0.431, 95% CI 0.211–0.880, p = 0.021).

Conclusions: LOS was associated with increased neutrophil and decreased creatinine. Understanding these factors can assist in optimizing care and resource allocation in hospitals during health crises.

Keywords: COVID-19; Length of hospital stay; Jordan.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) emerged in the Chinese city of Wuhan, and due to its high transmissibility has spread rapidly around the globe[1-3]. COVID-19's ongoing pandemic has caused significant risk to healthcare globally[4, 5].

Healthcare institutions throughout the globe are experiencing an extraordinary severe crisis due to their inadequate capability. Early management optimization may enhance the prognosis in individuals at high risk of acquiring the most severe manifestations, although initial detection of poor prognosis and risk classification is still a problematic issue[6]. As a result, accurate biomarkers or risk evaluation frameworks that can indicate a severe prognosis sooner are required to assist physicians in making decisions about the most suitable treatment strategy to achieve appropriate healthcare for each individual[7].

COVID-19 pandemic has caused substantial socioeconomic implications worldwide[8]. As COVID-19 pandemic persists, governmental healthcare and commercial organizations, and individuals are examining various ways to deal with it[9]. Early diagnosis and

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predictive modelling may improve the available therapeutic choices, leading to better clinical results and allowing limited resources to be used more effectively to combat COVID-19 and any future pandemic[10]. To mitigate the significant risk of mortality and optimize patient outcomes, it is imperative to develop therapeutic strategies that precisely predict both the risk of death and the length of hospital stay (LOS)[11]. Simultaneously, the exponential spread of infections necessitates an increase in working hours for healthcare workers (HCWs), which poses serious threats to their mental and physical health, underscoring the critical need for interventions that ensure their safety and well-being [12]. The aim of the current study is to evaluate risk factors associated with prolonged LOS of COVID-19 patients to enhance healthcare services and minimize economic and work burden.

METHODOLOGY:

Study sample

This is a retrospective case-control study that was conducted at Istiklal Hospital. Istiklal Hospital is one of the authorized institutions in Amman, Jordan, for the isolation and care for COVID-19 patients. 172 patients were hospitalized from January 13 until August 16, 2021, of which 89 patients were excluded due to either highly missing data or death. Ages of the enrolled participants were ranging from (1-93) years old. The severity of Covid-19 risk experienced by participants was estimated utilizing the Centers for Disease Control and Prevention (CDC) categorization (Centers for Disease Control and Prevention, 2021) [13].

Measurements

During their stay at Istiklal Hospital in Amman, Jordan, COVID-19 patients underwent a wide range of laboratory tests to check their health indicators. These tests included complete blood count (CBC), liver and kidney functions, electrolytes, lactate dehydrogenase (LDH) and C-reactive protein (CRP). To make sure the data was consistent and accurate, all tests were carried out with standardized equipment in the hospital's clinical lab. This careful setup ensured that the data gathered was reliable and helped meet the study's goals effectively.

Statistical Analysis

Continuous variables and categorical variables were described by medians (interquartile, IQR) and frequencies (percentages, %). Patients were grouped based on the sample median LOS (>7 and ≤7 days) to anticipate factors and variables that may impact LOS. For each predictor variable, bivariate binary logistic regressions were performed to calculate crude odds ratios (CODs). To correct for potential confounders and determine the unique association of each predictor with LOS, variables with a p-value of less than 0.25 in the bivariate binary logistic regressions (14 variables) were included in a forward stepwise multivariate binary logistic regression analysis. Adjusted odds ratios (AORs) were computed for each of the significant predictors in the multivariate binary logistic regression. Multicollinearity between different predictors was evaluated by applying chi-square and Pearson's correlations. Nagelkerke R Square was examined to determine the most suitable model that explains the most variance in the outcome variable, while minimizing the number of predictors to avoid model overfitting. All statistical analyses were conducted using IBM SPSS, version 22. Finally, a p-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 83 patients were included in the final analysis. Males made up most of the research participants, 53(63.9 %). The median age of the research participants was 58 years (IQR = 44-73). **(Table1)**

Table1. Sociodemographic characteristics of diagnosed COVID-19 study population

Variables	Category	Frequency	%
Gender	Male	53	63.9
	Female	30	36.1
Male age (years)	Median (IQR)	58 (42.50-72)	
Female age (years)	Median (IQR)	58.50 (47.75-73)	
Age (years)	Median (IQR)	58 (44-73)	
Age group (years)			
	1-19	2	2.4
	20-39	11	13.3
	40-59	33	39.8
	≥60	37	44.6
LOS for males (days)	Median (IQR)	8 (5-10.50)	
LOS for females (days)	Median (IQR)	6 (4.75-11.25)	
LOS (days)	Median (IQR)	7 (5-11)	
Hospital LOS	<7 days	35	42.2
Hospital LOS	≥7 days	48	57.8
Current Smoking	YES	4	4.8
	NO	79	95.2

LOS: length of stay, IQR: interquartile range

Fever and cough were experienced by 54 (65.1%) and 70 (84.3%), patients respectively. Most patients (80.7%) had respiratory difficulties. In addition, as indicated in **(Table2)**, the median SpO2, respiratory rate, and body temperature at admission were 95 (IQR = 92–96), 19 (IQR = 18–20), and 36.90 (IQR = 36.60–37.30) respectively.

Table2. Clinical, risky behavioral characteristics of COVID-19 patients

Variables	Category	Frequency	%
Fever	YES	54	65.1
	NO	29	34.9
Cough	YES	70	84.3
	NO	13	15.7
Difficulty of breathing	YES	67	80.7
	NO	16	19.3
Symptoms prior to admission	Days	7 (3-10)	
Temperature (°C)	Median (IQR)	36.90 (36.60-37.30)	
Respiratory rate	Median (IQR)	19 (18-20)	
Heart rate	Median (IQR)	85 (77-96)	
Spo2	Median (IQR)	95 (92-96)	
MAP	Median (IQR)	95.33 (88.33-100.33)	
Presentation with comorbidities	YES	40	51.8
	NO	43	48.2
Degree of Covid-19 risk			
	High-Risk Group	37	44.6
	Medium-Risk Group	13	15.7
	Low-Risk Group	33	39.8
Covid-19 patients' laboratory findings			
CRP	Median (IQR)	70 (23.94-122)	
D-Dimer	Median (IQR)	0.57 (0.30-0.89)	
LDH	Median (IQR)	458 (379.90-542.20)	
Ferritin	Median (IQR)	697.50 (425-1309)	
Urea	Median (IQR)	48.20 (34.60-68.30)	
Creatinine	Median (IQR)	0.79 (0.65-1.02)	
eGFR	Median (IQR)	90.90 (57.90-126.70)	
Uric acid	Median (IQR)	4.70 (3.80-5.70)	

Variables	Category	Frequency	%
Hemoglobin	Median (IQR)	14.10 (12.40-14.90)	
Red blood cells count	Median (IQR)	5.04 (4.66-5.34)	
Platelets count	Median (IQR)	367 (275-441)	
White blood cells count	Median (IQR)	10.70 (7.63-14.54)	
Neutrophils count	Median (IQR)	88 (82.50-92)	
Lymphocytes count	Median (IQR)	12 (8.50-18)	
Monocytes count	Median (IQR)	6 (4.60-8)	

MAP: mean arterial blood pressure, LDH: lactate dehydrogenase, CRP: c-reactive protein,

SPO2: saturation of peripheral oxygen, IQR: interquartile range, eGFR: Estimates glomerular filtration rate

The median LOS in the hospital was 7 (IQR=6-8) days. Patients with an elevated neutrophil count and deceased creatinine level significantly correlated with a more extended LOS. A one-unit rise in the neutrophil count was associated with a 14.4% increase in the likelihood of a prolonged LOS (AOR = 1.144, 95% CI: 1.059-1.236). The odds of having a one-unit rise in creatinine decreased LOS by 56.9% (AOR = 0.431, 95% CI: 0.221-0.880). Nagelkerke R Square revealed that the model explained 33% of the variation in LOS, the model's sensitivity and specificity were 83.30 and 45.70%, respectively. **(Table3)**

Table3. Factors associated with prolonged hospital stay among COVID-19 patients

Variables	COR (95%CI)	P	AOR (95%CI)	p
Gender	2.045 (0.823-5.085)	0.124	-	
Admission CRP	1.005 (0.999-1.012)	0.124	-	
Symptoms-PTA	1.067 (0.965-1.181)	0.206	-	
Heart rate	1.022 (0.988-1.056)	0.210	-	
Respiratory rate	0.925 (0.812-1.053)	0.237	-	
Admission SPO2	0.880 (0.761-1.019)	0.087	-	
General Weakness	2.035 (0.840-4.930)	0.115	-	
Chest Pain	2.025 (0.759-5.405)	0.159	-	
Vitamins	2.343 (0.791-6.943)	0.125	-	
LDH	1.002 (1.000-1.004)	0.132	-	
Creatinine	0.695 (0.390-1.241)	0.219	0.431 (0.210-0.880)	0.021
Platelets	1.003 (1.000-1.007)	0.079	-	
Neutrophils	1.111 (1.038-1.190)	0.002	1.144 (1.059-1.236)	0.001
Monocytes	0.858 (0.738-0.999)	0.049	-	

Omnibus $\mathbf{x}^2$ (2) =23.368, p < 0.001, R^2 = 0.33 (Negelkerke), LDH: lactate dehydrogenase, CRP: c-reactive protein, PTA: prior to admission, SPO2: saturation of peripheral oxygen

DISCUSSION

Many nations' medical resources were depleted during the COVID-19 pandemic. Understanding hospital LOS and variables that influence it may help physicians make better decisions regarding patient selection, design solutions to limit LOS, and ultimately reduce resource consumption. This retrospective case-control study, characteristics and variables linked to LOS during hospitalization in patients with COVID-19 we evaluated.

Comparative Analysis of LOS

Patients in this study varied in age from 1 to 93 years old, with males accounting for more than half (63.9%) of the patients. Similar to research conducted in Beijing and other parts of China[14], COVID-19 might affect people of all ages from both genders. The LOS in our patients was 7 days (IQR = 6–8); in agreement with previous studies conducted in the United States and other European nations, in which the LOS was 7–8 days[15]. However, a research in Sichuan province, China, showed a broader range of in-hospital stay of 19 (IQR: 3–41) days. This disparity might be explained by differences in COVID-19 severity and/or management strategies[16, 17].

Key Variables Affecting LOS

Bivariate binary logistic regression revealed that neutrophil count showed a higher prevalence of prolonged LOS among COVID-19 patients, it also revealed that no significant relationship between LOS and other characteristics previously linked to LOS or severe COVID-19 prognosis, such as age, gender, symptoms, and comorbidities were found in the present study[18-22]. However, because of differences in data collection methods (e.g., interviews vs. medical records), disease severity assessments used as outcomes (e.g., hospitalization, ICU admission, mechanical ventilation, or death), and how underlying medical conditions are distributed among various populations, some variation between the findings of this study and previous studies investigating risk factors for prolonged LOS are expected[23].

The results of the present study revealed that increased levels of inflammation-related variables, such as neutrophil, was shown to be associated with poor outcomes. Chemokines and cytokines are mostly produced by neutrophils. It has been found that elevated neutrophilia levels were prevalent in both the peripheral blood and lungs of SARS and MERS patients. These findings show that elevated neutrophils may be a prevalent feature of coronavirus infection. The monitoring of neutrophils count should be increased during hospitalization since numerous infections are usually associated with this disease and may result in a poor outcome[24]. Furthermore, clinical research should be more cautious and extensive, taking into consideration the patients' usage of corticosteroids due to their contradictory effects on neutrophilia.

In the other hand, an increase in creatinine level was linked to a lower prevalence of prolonged LOS. Low baseline blood creatinine levels were associated with an elevated mortality risk in a large retrospective cohort study of 11291 critically ill patients admitted to intensive care units. As a result, decreased serum creatinine levels may be associated with a prolonged LOS among COVID-19 patients [25]. A larger sample size of people from diverse backgrounds in Jordan and throughout the world might help us better understand what patient features, drugs, and underlying medical illnesses are connected to hospitalization and poor outcomes in individuals with COVID-19.

Implications for Healthcare Strategies

This study explored evidence for anticipating inpatient bed needs in an innovative public health intervention setting by investigating the duration of hospitalization, an important but underestimated parameter, and its related characteristics among COVID-19 patients hospitalized in Istiklal hospital. The association between neutrophil counts and prolonged LOS, emphasize the importance of monitoring neutrophils count in addition to other inflammatory biomarkers. Understanding these factors better helps us optimize patients care and may help decision-makers with strategies for increasing the performance of the healthcare system and allocating resources.

Limitations of the Study

This study has numerous limitations as follows: First, because of the change of testing procedures and demographics of infected people throughout the pandemic and participants' socioeconomic background and underlying health state, our results are particular to this population and may not be generalizable to other populations. Second, since

this is single-centered research with small sample size, our interpretation of the analytic findings may be limited. Therefore, future multicentral research with larger sample size can confirm the generalizability of the study results. Third, not all laboratory values for all patients were obtained due to the retrospective research structure. Missing data were replaced with the corresponding median value. Prospective studies with more rigorous data collection protocols are needed to minimize data gaps and improve the accuracy of the findings. Furthermore, reporting mistakes are likely to be non-discriminatory among research respondents. Finally, since a limited proportion of respondents reported numerous demographics or social factors, particular underlying medical problems, and treatments, our ability to draw inferences regarding their relationships with hospitalization was restricted; this also made it impossible to thoroughly investigate and compensate for the relationship of potential risk concerns. Future studies should prioritize comprehensive data collection methodologies, including standardized reporting guidelines, to ensure thorough documentation of demographics, social factors, medical history, and treatments, thereby facilitating more robust analyses and mitigating potential biases in assessing their relationships with hospitalization.

CONCLUSION

Identifying patients at high risk of developing poor clinical outcomes is a top concern for healthcare providers amidst the COVID-19 crisis. The study found that elevated neutrophil count was possible predictor of longer LOS. Patients with extended hospital stays typically require more intensive and prolonged medical interventions. Such scenarios necessitate increased use of pharmacological treatments, more frequent diagnostic testing, and greater utilization of non-pharmacological support measures (e.g., oxygen therapy, nutritional support). Consequently, these patients are expected to consume more pharmacological and non-pharmacological supplies while receiving medical treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethical Approval

Ethical approval was obtained from Al-Zaytoonah University Ethics Committee. The research was carried out in accordance with Declaration of Helsinki guidelines and oregulations.

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فهم العوامل المؤثرة على مدة الإقامة في المستشفى بين مرضى كوفيد-19: دراسة استعادية للحالات والشواهد في الأردن

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ملخص

الهدف :مرض فيروس كورونا 2019 (كوفيد-19) هو مرض تنفسي حاد شديد مرتبط بعدوى الجهاز التنفسي العلوي) فيروس كورونا المستجد .(SARS-CoV-2 - إن تقييم العوامل التي تؤثر على مدة الإقامة في المستشفى والمضاعفات لدى مرضى كوفيد-19 يمكن أن يساعد في تقليل الأحداث غير المرغوب فيها بشكل فعال وآمن. ولذلك، هدفت هذه الدراسة إلى تقييم العوامل التي تؤثر على مدة الإقامة في المستشفى لدى مرضى كوفيد-19.

المرضى والطرق :دراسة استعادية حالة-شاهد تشمل المرضى في عمان، الأردن، الذين تم تأكيد إصابتهم بفيروس كوفيد-19 مختبريًا وتم إدخالهم إلى المستشفى بين 13 يناير و16 أغسطس 2021. تم الحصول على المعلومات الديموغرافية، والعلاج السريري، وبيانات المختبر من سجلاتهم الطبية.

النتائج :كانت مدة الإقامة في المستشفى لـ 83 مريضًا المتوسطة 7 أيام (الفترة بين الربيع 6-8 أيام). للتحقيق في المتغيرات المترابطة مع مدة الإقامة الطويلة، تم استخدام الانحدار اللوجستي الثنائي الثنائي المتغير والمتعدد المتغيرات. تم حساب نسب الأرجحية المعدلة (AORs) وفترات الثقة 95 .(CIs) %كان متوسط عمر المرضى 58 سنة. وكان حوالي نصف المرضى في المجموعة عالية المخاطر (44.6%). وفقًا لتحليل الانحدار اللوجستي الثنائي المتعدد المتغيرات على الـ 83 مريضًا، تم العثور على أن أحد العوامل التي تعتبر عامل خطر لزيادة مدة الإقامة الطويلة هو عدد العدلات (AOR = 1.144، 95% CI 1.059–1.236 ، p = 0.001). في المقابل، تم العثور على أن زيادة مستوى الكرياتينين يعتبر عامل وقاية ضد زيادة مدة الإقامة الطويلة(AOR = 0.431 ، 95% CI 0.211–0.880 ، p = 0.021).

الاستنتاجات :كانت احتمالية الإقامة لفترة طويلة في المستشفى مرتبطة بزيادة عدد العدلات، في حين أن زيادة وحدة واحدة في مستوى الكرياتينين كانت مرتبطة بتقليص مدة الإقامة في المستشفى.

الكلمات الدالة :كوفيد-19، مدة الإقامة في المستشفى، الأردن

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Enhanced Platelet Activation Induced by Palbociclib Treatment in MCF-7 Breast Cancer Cells

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ABSTRACT

Background and aim: The crosstalk between platelets and cancer cells is bidirectional. Cancer cells can activate platelets, a process known as "tumor cell-induced platelet aggregation". On the other hand, platelets provide essential assistance to tumor cells by helping them adhere to blood vessels, facilitating their escape from the bloodstream, and enabling their spread to distant tissues. This study investigates the effect of Palbociclib, an FDA-approved cyclin-dependent kinase 4 and 6 inhibitor, on breast cancer-cell-induced platelet activation.

Method: Platelet activation, as indicated by the expression of CD62P (P-selectin), was assessed by flow cytometry. In addition, platelet attachment to MCF-7 cancer cells was studied using confocal microscopy.

Results: Palbociclib's half-maximal concentration (IC_{50}) was found to be 19.54 µM after 72 h. About 36.9% $\pm$ 0.98 of platelets were activated by untreated MCF-7. Pretreatment of MCF-7 cells with Palbociclib (9.75 µM equivalent to 1/2 IC_{50}) increased platelet activation significantly by 63.3% $\pm$ 8.85 ($P<0.01$) while 4.87 µM Palbociclib (1/4 IC_{50}) increased platelet activation by 43.0% $\pm$ 2.83 with no significant difference compared to untreated cells. Confocal microscopy results suggest the presence of a direct interaction between breast cancer cells and platelets.

Conclusion: Palbociclib increased tumor-induced platelet activation in MCF-7 hormone-positive breast cancer cell line.

Keywords: Confocal microscopy; Flow cytometry; MCF-7; Palbociclib; Platelets; P-selectin**.**

INTRODUCTION

The bidirectional interaction between platelets and tumor cells was reported since 1968. Platelets can be activated by cancer cells both *in vitro* and *in vivo*, a process known as "tumor cell-induced platelet aggregation"[(1)]. Beyond regulating tumor growth, platelets play an essential role in metastasis [(2)]. They adhere to the surface of cancerous cells shielding them from shear stress and hindering immune system recognition and attack [(3)]. Furthermore, platelets facilitate the adherence of tumor cells to blood vessels enabling them to escape from the bloodstream and spread to distant organs [(4)]. Moreover, platelet granules contain substances that aid in the metastasis and promote tumor vascularization including P-selectin (CD62P) and CD63 which are exposed on the platelet surface after activation [(5)].

Breast cancer is the most frequent cancer in women and the leading cause of cancer death in females worldwide (6-10). According to World Health Organization (WHO) estimate, one million women are newly diagnosed with breast cancer yearly. In Jordan, breast cancer ranks as the most prevalent malignancy and stands as the third most frequent cause of cancer-related mortality, after lung and colorectal cancers [(11)]. In 2015, food and drug

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administration (FDA) approved the first cyclin-dependent kinase 4 and 6 inhibitor (CDK4/6i), Palbociclib, as a first-line therapy for postmenopausal women with hormone positive/HER2 negative advanced or metastatic breast cancer to be used in combination with letrozole or fulvestrant [12].

A multicenter retrospective study conducted in the USA, which included 266 patients, found that the 1-year incidence of thrombosis in patients taking Palbociclib was 10.9%. Most thrombotic events recorded were venous (72%) and only 34% were arterial. This happened despite the cohort's aspirin use rate of 26%, which may have reduced some of the thrombotic risk [13]. In another study, Palbociclib had the highest 1-year cumulative incidence of thrombosis, followed by Ribociclib and Abemaciclib [13]. A higher proportion of patients undergoing treatment with Palbociclib and Ribociclib exhibited cardiovascular comorbidities, notably hypertension, in contrast to those treated with Abemaciclib. Specifically, 30% of individuals who developed venous thromboembolism while using Palbociclib had cardiovascular conditions[14]. In a retrospective study involving 424 patients, venous thromboembolic events occurred in 9% of subjects (mainly receiving Palbociclib). Deep venous thrombosis alone was found to be the most common presentation (47.4%), followed by visceral vein thrombosis (21.1%) and pulmonary embolism (18.4%) [15]. A single-center audit of 64 individuals treated with Palbociclib over five months in an Irish tertiary referral hospital recorded seven venous thromboembolic events comprising 11% of the studied group [16], whereas no increased risk of pulmonary embolism was found when comparing new users of Palbociclib–fulvestrant with patients using fulvestrant alone [17].

Due to the multifactorial etiology of cardiovascular diseases, causative evidence concerning the effect of CDK4/6i(s) in general and Palbociclib in particular on thrombotic events is hard to achieve. Understanding how Palbociclib increases the risk of thrombosis could aid in implementing preventive measures during treatment. The current study examined the impact of Palbociclib on tumor-induced platelet activation in a hormone-positive breast cancer cell line through *in vitro* experiments utilizing flow cytometry and confocal microscopy.

MATERIALS AND METHODS

Ethical consideration

This study was approved by the research ethical committee at Al-Ahliyya Amman University (ethical approval number IRB: AAU/5/14/2021-2022). Written and verbal informed consents were taken from all study participants. Blood (2.5 mL) was collected from 4 healthy females between 19 and 30 years old. To prevent platelet activation during blood drawing, a 21-gauge needle was used. The first 2 mL of blood drawn were discarded to exclude the effect of mechanically activated platelets. Whole blood was collected in sodium citrate tubes. Participants' exclusion criteria included pregnancy, oral contraceptive use, previously identified disease, smoking, and consumption of any medication in the previous week before the experiment. Blood was collected between days 1 and 10 of the menstrual cycle since low levels of estrogen and progesterone in blood exist during this period. To prepare platelets-rich plasma (PRP), whole blood samples were centrifuged at 200x g for 15 minutes.

Cytotoxicity assay

MCF-7 cell lines were kindly provided by The University of Jordan and cultured in accordance with established procedures and techniques. The MTT (3- [4, 5-dimethyl-2-thiazolyl] - 2, 5-diphenyl tetrazolium bromide) kit (Thermo scientific, USA) was used to assess cell cytotoxicity as previously described [18]. MCF-7 (passage 17) cells were cultured in RPMI media to which 10% (v/v) heat-inactivated fetal bovine serum, penicillin (50 mg/mL), and streptomycin (50 mg/mL) were added and maintained at 37 °C under 5% CO_2 and 95% humidity. MCF-7 cells (10^4 cells) were planted into 96-well plates and incubated for 24 hours then treated with Palbociclib

(Tocris Bioscience, UK) dissolved in sterile distilled water for 72 hours. Cells without any drug treatment were used as a control.

Co-culturing platelets with cancer cells

MCF-7 (Passage number: 18) cells were seeded onto 24-well plates at 10^5 cells/well seeding density and incubated in 1 mL media for 24 hours at 37 °C and 5% CO_2 for cell adherence and monolayer formation. After 24 hours, MCF-7 cells were incubated with Palbociclib at 9.75 µM (1/2 IC_{50}) and 4.87 µM (1/4 IC_{50}) of Palbociclib in media for 72 hours at 37 °C. After 72 hours of drug treatment, the media in the wells was removed, and cells were washed twice with phosphate-buffered saline (PBS). The remaining cells were then treated for 20 minutes with 200 µL diluted PRP in PBS (1:1 ratio). Then PBS was used to wash cells after incubation with PRP. The PRP and the PBS used for washing were centrifuged at 200× *g* for 5 minutes and the resulting pellet was re-suspended in 150 µL PBS buffer for double-labeling with mouse monoclonal CD42b antibody conjugated with phycoerythrin (PE) (Biotech/Novus USA-UK) (1:100 dilution) and mouse monoclonal CD62P antibody conjugated with fluorescein isothiocyanate (FITC) (Biotech/Novus USA-UK) using 1:100 dilution in the dark at room temperature for 10 minutes at 37 °C.

Flow cytometry

PRP alone was used to measure the auto-fluorescence of the plasma. Adenosine diphosphate (ADP, HART/UK) was incubated with PRP for 20 minutes and used as a positive control for platelet activation. Compensation controls used in platelets' population gating were prepared by incubating platelets in PRP with a single antibody (either anti-CD42 or anti-CD62P). The samples were then fixed with 1% paraformaldehyde for 10 minutes before being centrifuged at 200×*g* for 5 minutes and the supernatant was discarded. The pellet was gently re-suspended in 500 µL of PBS buffer, transferred into flow cytometry tubes, and kept at 4°C before data analysis.

Data was collected on the flow cytometer FACSCanto TM II (BD Biosciences, USA) at Cell Therapy Center (CTC), Jordan. All experiments were repeated in triplicates, each from a different donor of blood. A gating strategy was used to detect the percentage of positivity of CD62P expressed on platelets using a single histogram gated on CD42b-positive cells, a specific marker for platelets, and CD62P positive cells (activated platelets). Mean fluorescence intensity was measured and the percentage activation of platelets was calculated using the formula:

% activation = Q2/(Q1+Q2) where Q1 is the population of platelets stained with PE-labeled CD42 antibody and Q2: is the population stained with both FITC-labeled CD62P and PE-labeled CD42 antibody.

Study of platelet-cancer cell interaction using confocal microscopy

Coverslips were fixed in wells by adding alcohol for 20 minutes. Then, MCF-7 cells (passage: 25) were seeded onto 6-well plates at $60*10^3$ cells/well and incubated in 1ml media for 24 hours at 37 °C and 5% CO_2 for cell adherence and monolayer formation. After 24 hours, MCF-7 cells were incubated with 19.5 µM of Palbociclib (IC_{50}) at 37 °C. After 72 hours of Palbociclib treatment, the media in the wells was removed and cells were washed with PBS. The remaining cells were then treated with 400 µL of PRP for 20 minutes after dilution of PRP with PBS in 1:1 ratio. PRP was then removed and the two antibodies (FITC-labeled CD62P (1:100 dilution) and PE-labeled CD42 (1:100 dilution) were added to wells. The cells were then fixed with 4% paraformaldehyde for 10 minutes before adding ammonium chloride washing buffer for 10 minutes at room temperature. DAPI (4′,6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA, was added in 1:1500 dilution for 10 minutes. After removing DAPI, the wells were washed. Finally, slides were examined using a confocal immunofluorescent microscope LSM 780 microscopy ZEISS (Germany) at CTC using 63x objective lens.

Statistical analysis

GraphPad Prism version 8 was used to perform

statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used. Results were considered statistically significant at p <0.05

RESULTS

Cell cytotoxicity assay

The half maximal concentration (IC_{50}) value for Palbociclib against MCF-7 cell line after 72 hours was 19.54 ± 4.97 µM.

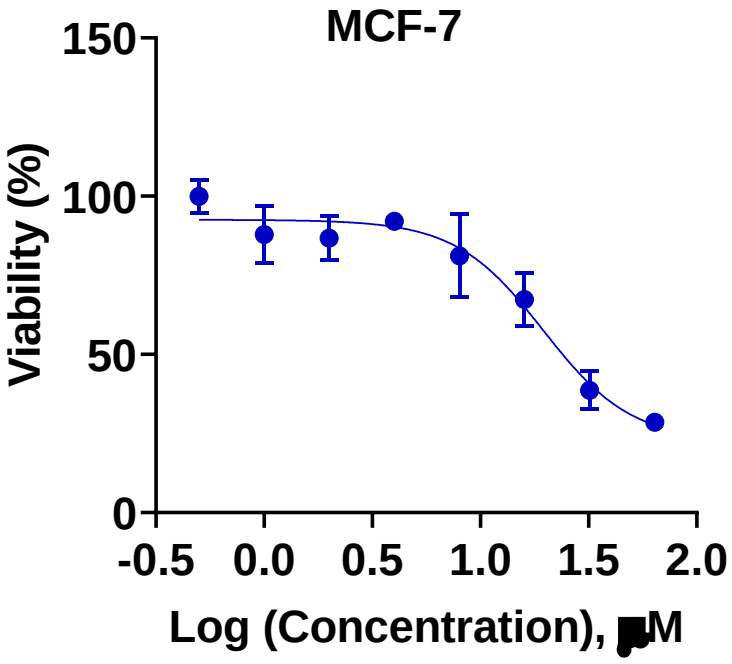

Figure 1: Dose-dependent cytotoxicity assay of Palbociclib on MCF-7 cell line at 72 hours. Each concentration was performed in triplicate.

Percentage change in CD62P expression on platelets co-cultured with MCF-7 cell line.

ADP-treated platelets (positive control) had 84.4% ± 0.14 expression of CD62P (Figure 2A). About 36.9% ± 0.98 of platelets co-cultured with untreated MCF-7 were activated (Figure 2B). Platelets exposed to MCF-7 cells treated with 9.75 µM (1/2 IC_{50}) or 4.87 µM (1/4 IC_{50}) Palbociclib showed 63.3% ± 8.85 and 43.0% ± 2.83 expression of CD62P on their surface, respectively (Figure 2C-D).

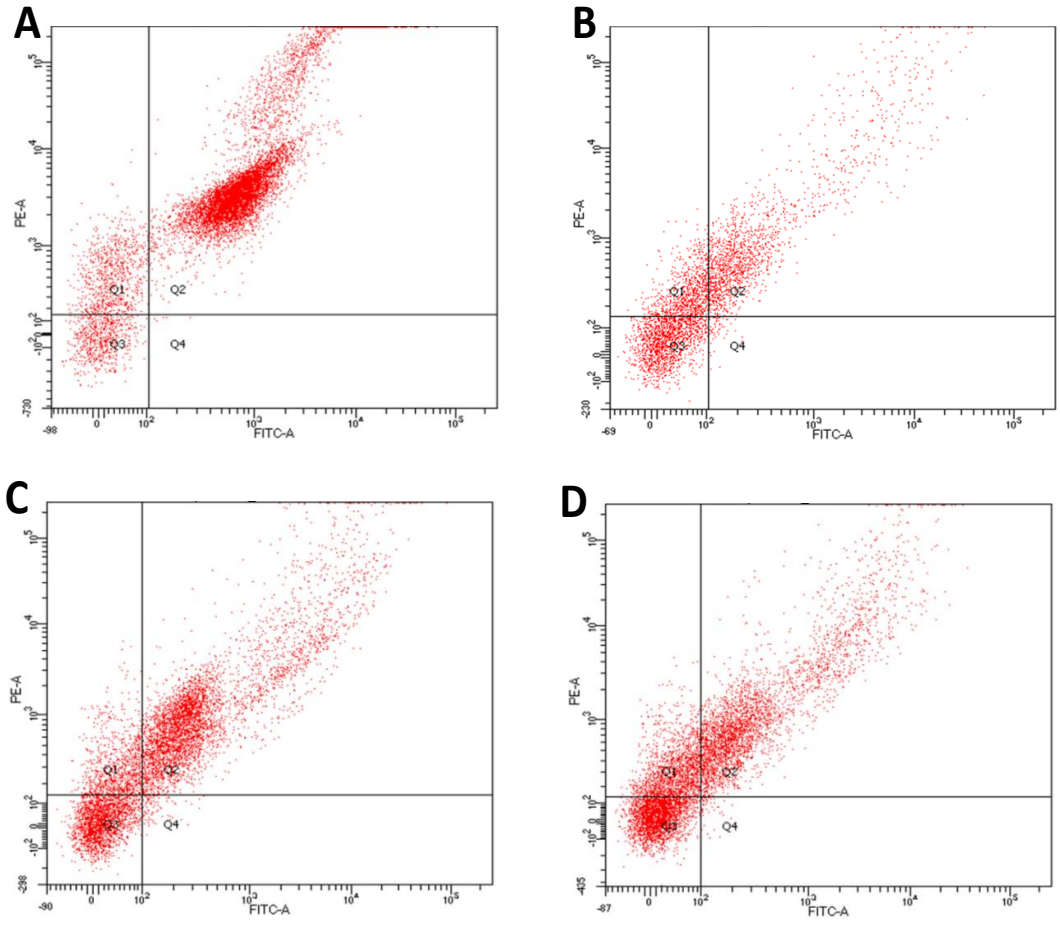

Figure 2: Expression of the activation marker P-selectin (CD62P) on platelets co-cultured with MCF-7 as studied by flow cytometry. The Y-axis represents fluorescence intensity of PE-labeled CD42 while X-axis represents FITC-labeled CD62P. The percentage activation of platelets was calculated using the formula: % activation = Q2/(Q1+Q2) where Q1 is the population of platelets stained with PE-labeled CD42 antibody and Q2 is the population stained with both FITC-labeled CD62P and PE-labeled CD42 antibodies. (A) Platelets exposed to ADP (positive control). (B) Platelets exposed to untreated MCF-7. (C) Platelets exposed to MCF-7 treated with 1/2 IC_{50} (9.75µM) of Palbociclib, (D) Platelets exposed to MCF-7 treated with 1/4 IC_{50} (4.87µM) of Palbociclib.

A significant difference in platelets' activation was observed between platelets co-cultured with untreated MCF-7 cells and platelets co-cultured with MCF-7 treated with Palbociclib (9.75 µM doses) ($P<0.01$) but not with the lower dose of Palbociclib (4.87µM). Also, a significant difference was found between platelets incubated with untreated MCF-7 and ADP-treated platelets ($P<0.001$) (Figure 3).

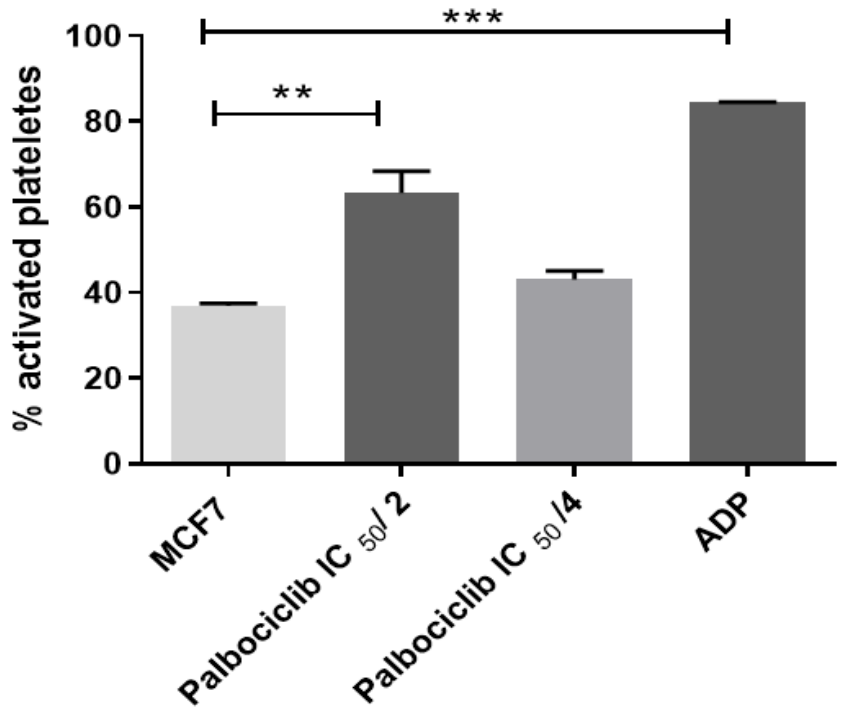

Figure 3: Platelet activation percent in flow cytometry upon co-culture of platelets with untreated MCF-7 cells, Palbociclib-treated cells or ADP (20 µM), used as a positive control, for 20 minutes. * P≤0.05, ** P≤0.01, ***P≤0.001

Confocal microscopy

Confocal microscopic examination revealed that platelets were attached to untreated MCF-7 cells and can be seen as red dots after staining with PE-labelled CD42 antibody (Figure 4C). However, this antibody can't distinguish activated platelets from inactivated platelets. Therefore, FITC-labelled CD62P antibody was used to stain activated platelets as depicted in (Figure 4A) where a clear expression of CD62P on the platelets' surface attached to MCF-7 was seen as green dots, indicating that platelets were activated.

When MCF-7 cells were treated with Palbociclib and then incubated with platelets, a pronounced increase in the intensity of green color was observed indicating that CD62P is expressed on platelets' surface (Figure 4E). Attachment of platelets to MCF7 cancer cells was evident as indicated by the presence of intense red and co-localization with MCF7 cells (Figure 4F, G).

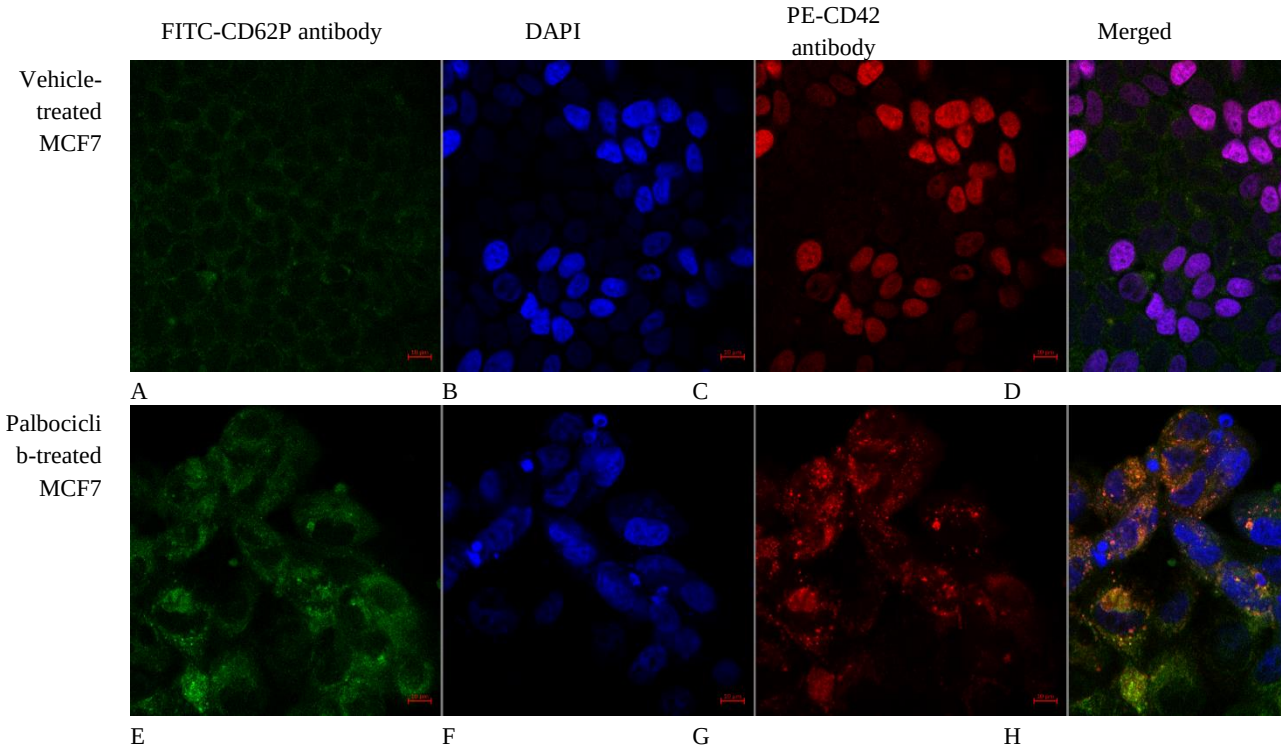

Figure 4: Attachment of platelets to MCF-7 breast cancer cells. (A-D) Platelets incubated with untreated MCF-7 cells stained with FITC-CD62P antibody (A), DAPI (B) or PE-CD42 antibody (C). (D) Represents merged A-C images. (E-H) Platelets incubated with 19.54 µM Palbociclib-treated MCF-7 stained with FITC-CD62P antibody (E), DAPI (F), or PE-CD42 antibody (G). (H) Represents merged E-G images (scale bar: 10µM).

DISCUSSION

Platelets can be activated *in vitro* and *in vivo* by different cancer cell types, a process known as "tumor cell-induced platelet aggregation" [(1)]. Important granular membrane molecules, including CD62P and CD63, become exposed on the platelet's cell surface after activation, aiding tumor development and metastasis [(19)]. In the present study, co-culturing MCF-7 with platelets activated them. This activation is indicated by the expression of CD62P on their surface. Earlier studies reported similar findings [(20)]. In a recent study, both MDA-MB-231 and MCF-7 cells increased platelets activation through the secretion of ATP from cancer cells. When platelets and MCF-7 cells were co-incubated, there was a four-fold increase in ATP release from platelet's granules in the case of MCF-7 cells [(21)]. In the present study, ATP release from cancer cells was not investigated. However, activation by direct contact was evident using confocal microscopy.

In our study, Palbociclib pre-treatment of MCF-7 cells increased their activation to platelets, as indicated by the presence of the activation marker CD62P on their surface. Similar results were obtained by other studies in which CD62P increased on the surface of platelets stimulated by cancer cells compared with resting platelets [(19, 22)].

According to reports, patients treated with Palbociclib had a relative risk for thromboembolic events (13). The pathophysiology of thrombotic events in cancer is complicated, and the individual patient's risk for

thrombotic events is affected by a multifactorial interplay between the person's cardiovascular risk and comorbidities, the specific neoplasm, and the treatment regimen [23]. The exact mechanisms underlying cancer therapy-related thrombosis remain uncertain. It is believed that anticancer medications have the potential to activate or disturb the endothelium, which could be a primary mechanism [24]. Additionally, these drugs may reduce the levels of anticoagulant substances or increase the levels of procoagulant substances, activating the coagulation process. Furthermore, anticancer drugs can directly or indirectly stimulate platelets [25]. The findings of this study confirm that Palbociclib-treated, hormone-positive MCF-7 cells exhibit increased platelet activation due to direct interactions between the cancer cells and platelets.

In the present study, ADP was used as a positive control. The percentage of platelets expressing the activation marker CD62P measured by flow cytometry was 84.4% after platelet incubation for 20 minutes with ADP (20 µM) at room temperature. When PRP was incubated with 10 µM ADP for 8 minutes at room temperature, CD62P expression, measured by flow cytometry, was about 59% (26). Another study reported a significant increase in platelet activation after incubation with ADP for 15 minutes, with 54.7% activation [27]. The degree of platelet activation by ADP can be influenced by varying ADP concentrations and different incubation periods.

It is well-known that platelets and cancer cells interact bi-directionally to promote tumor development and metastasis (6). The direct platelet-tumor cell contact depends on many factors facilitating interaction, including adhesion molecules, selectins and integrins (28). High platelet activation was reported by direct contact assessed by confocal microscopy as indicated by the increase in platelet activation markers PAC-1 and P-selectin (29). In the present study, platelet activation was clear as seen using confocal microscopy where platelets incubated with MCF-7 expressed the activation marker CD62P. In a previous study, co-incubation of platelets with MCF-7 showed that platelets promoted the invasion of these cells after direct contact with platelets. Labeling platelets with P-selectin antibody and studying them with flow cytometry, confirmed the enhancement of contacting ability between MCF-7 cells and platelets via surface integrin α2β1 (30). In another study, platelet shape change was visible after co-culturing with MCF-7, indicating at least partially activated platelets when platelets were stained by CD42a and PKH67 antibodies and studied with a confocal microscope [31]. This agrees with our results in which platelets were adherent to MCF-7 and activated.

Clinical trials have reported an increased incidence of thrombotic events associated with Palbociclib therapy [9]. This study elucidates a mechanism by which Palbociclib enhances platelet activation by increasing tumor-cell enhanced platelet activation. Whether this effect translates to in vivo settings remains to be verified. Understanding the mechanisms through which Palbociclib heightens thrombotic risks could facilitate the implementation of preventive measures during Palbociclib treatment, potentially involving the prescription of anti-platelet medications for patients. Further clinical investigations are warranted to explore this issue.

CONCLUSION

Treating hormone-positive breast cancer cell line MCF-7 with Palbociclib increased the activation of platelets. The effect of Palbociclib was confirmed by confocal microscopy in which platelets directly interacted with cancer cells. This direct interaction is responsible, at least partially, for platelet activation by cancer cells. Understanding how Palbociclib increases the risk of thrombosis could aid in implementing preventive measures during treatment and the use of antiplatelet medications for patients.

Conflicts of interest statement

The authors declared no conflict of interest in this study.

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زيادة تنشيط الصفائح الدموية الناتج عن العلاج بالبالبوسيكليب في خلايا سرطان الثدي MCF-7

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ملخص

الخلفية والهدف: ان التواصل المتبادل بين الصفائح الدموية وخلايا السرطان ثنائي الاتجاه. إذ يمكن للخلايا السرطانية تنشيط الصفائح الدموية، وهي عملية تُعرف باسم "تراص الصفائح الدموية الناجم عن الخلايا السرطانية". من ناحية أخرى، توفر الصفائح الدموية دعمًا أساسيًا للخلايا السرطانية من خلال مساعدتها على الالتصاق بالأوعية الدموية، مما يسهل هروبها من مجرى الدم وانتشارها إلى الأنسجة البعيدة. تبحث هذه الدراسة في تأثير عقار بالبالبوسيكليب، وهو مثبط لإنزيمات كيناز المعتمدة على السيكلين 4 و6 معتمد من قِبل إدارة الغذاء والدواء (FDA)، على تنشيط الصفائح الدموية الناجم عن خلايا سرطان الثدي.

الطريقة : تم تقييم تنشيط الصفائح الدموية من خلال قياس تعبير CD62P (P-selectin) باستخدام جهاز التدفق الخلوي. بالإضافة إلى ذلك، تم دراسة التصاق الصفائح الدموية بخلايا سرطان الثدي MCF-7 باستخدام المجهر متحد البؤر.

النتائج: تم تحديد التركيز المثبط للنصف (IC_{50}) لعقار البالبوسيكليب بقيمة 19.54 ميكرومول بعد 72 ساعة. تم تنشيط حوالي 36.9% ± 0.98 من الصفائح الدموية بواسطة خلايا MCF-7 غير المعالجة. المعالجة المسبقة لخلايا MCF-7بتركيز 9.75 ميكرومول من البالبوسيكليب ما يعادل (1/2 IC_{50}) أدى إلى زيادة تنشيط الصفائح الدموية بشكل ملحوظ بنسبة 63.3% ± 8.85 (P<0.01) بينما زاد تركيز 4.87 ميكرومول (1/4 IC_{50}) من تنشيط الصفائح الدموية بنسبة 43.0% ± 2.83 دون فرق معنوي مقارنة بالخلايا غير المعالجة. تشير نتائج المجهر متحد البؤر إلى وجود تفاعل مباشر بين خلايا سرطان الثدي والصفائح الدموية.

الاستنتاج: عزز عقار بالبوسيكليب تنشيط الصفائح الدموية الناجم بواسطة خلايا سرطان الثدي الإيجابيه للهرمونات MCF-7.

الكلمات الدالة: المجهر متحد البؤر، جهاز التدفق الخلوي، MCF-7، بالبوسيكليب، الصفائح الدموية، .P-selectin.

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Hypolipidemic and Vasoprotective Potential of *Caralluma edulis*: A Histological and Biochemical Study

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ABSTRACT

Caralluma edulis (Apocynaceae) is well known for its medicinal properties, including antioxidant, anti-inflammatory, antimicrobial, and hypoglycemic activities, and has been used as a valuable remedy in various cultures. This scientific study aimed to validate the efficacy of *C. edulis* in lowering lipid profiles using two hyperlipidemic animal models: lipofundin-induced rabbits and fructose-induced rats. Lipofundin was administered intravenously at 2 mL/kg for 23 days, while fructose (25% w/v) was given for 28 consecutive days by dissolving it in drinking water to induce dyslipidemia and vascular dysfunction. The hydroalcoholic extract of *C. edulis* was orally administered (250 and 500 mg/kg) to experimental groups, while atorvastatin (10 mg/kg p.o.) was given only to the standard control group. Blood samples were collected to assess various biochemical parameters. Furthermore, histological examinations of liver and thoracic aorta tissues from fructose-fed rats were conducted, along with an evaluation of their vasorelaxant properties. The hydro-methanolic extract of *C. edulis* demonstrated dose-dependent hypolipidemic effects, significantly reducing serum cholesterol, triglycerides, and low-density lipoproteins at a dose of 500 mg/kg in both models, comparable to atorvastatin. Additionally, the hydroalcoholic extract exhibited significant endothelium-dependent vasorelaxant activity and hepatoprotective effects in fructose-fed rats. *C. edulis* also displayed antioxidant potential through free radical scavenging activity. These findings suggest that *C. edulis* possesses hypolipidemic and vasoprotective properties, likely attributed to its active pharmacological constituents, supporting its traditional use.

Keywords: Lipofundin; fructose-induced hyperlipidemia; atorvastatin; vasorelaxant studies; serum cholesterol.

1. INTRODUCTION

Cardiovascular diseases are the prominent cause of death in human being throughout the world [1] and is estimated to cause more or less 16.7 million deaths per year. In Pakistan, cardiovascular arterial disease affects 26.9% of the population, with a more pronounced incidence among women at 30% compared to men at 23.7% [2]. The prevalence of these diseases can be recognized by a number of factors such as alcohol consumption, smoking, sedentary lifestyle, consumption of high-calorie diets, aging, and genetic predisposition [3]. These factors contribute to the development of lipid and lipoprotein abnormalities, including deficiencies or overproduction. The relationship between elevated risks of

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coronary heart disease (CHD) and serum cholesterol levels was initially established in 1984, highlighting that a mere 1% reduction in serum cholesterol levels could potentially reduce the risk of CHD by 2% [4].

Hyperlipidemia stands as the primary cause of mortality in both developing and developed nations [5]. Plasma cholesterol levels exceeding >200 mg/dL serve as a significant risk factor, contributing to approximately 4.4 million deaths annually. Data from the National Health and Nutrition Examination Survey (NHANES) suggests that 11.7% of individuals aged 20-39 and 41.2% of those aged 40-64 exhibit elevated LDL levels. Moreover, hyperlipidemia affects 10.6% of individuals aged 20-39 and 47.7% of those aged 40-64 [6].

When lifestyle modifications prove ineffective in managing hyperlipidemia, pharmacological intervention becomes necessary to attain lipid-lowering objectives. Findings from a national survey (NEPTUNE II, 2003) indicated that 67% of 4885 hyperlipidemic patients successfully achieved their cholesterol-lowering targets. Moreover, data from national health and nutrition examination surveys revealed a decrease in the prevalence of hyperlipidemia, with statistics ceindicating that by 2002, no more than 17% of American patients would have elevated cholesterol levels [7]. Several lipid-lowering medications are available in the market to address hyperlipidemia. Currently, five primary classes of drugs are utilized in the management of this condition.

Research studies have shown that fructose consumption can lead to hypertension, insulin resistance, and hypertriglyceridemia. Upon ingestion and absorption in the gastrointestinal tract (GIT), fructose undergoes metabolism in hepatocytes to form acetyl CoA and glycerol-3 phosphate. Subsequently, the metabolites contribute to triglyceride synthesis, accelerating lipogenesis and resulting in the production and accumulation of VLDL (very low-density lipoproteins) and hepatic TG (triglycerides). Consequently, this process reduces insulin sensitivity and induces hepatic insulin resistance [8].

Synthetic drugs often pose a challenge due to their potential side effects outweighing their benefits. The initiation of drug therapy with HMG Co-A reductase inhibitors or fibric acid derivatives, which are inhibitors of CYP3A4 isozymes, has been linked to increased hospitalizations and gastrointestinal (GIT) issues [9]. Furthermore, the use of statins and fibrate combinations has been associated with elevated risks of renal failure and hepatic injury [10].

In contrast, herbal medicinal products constitute a significant portion, accounting for 57% of complementary medicine sales. A survey conducted in England in 1998 revealed that 22.1% of 5010 adults had purchased over-the-counter (OTC) herbal drugs [11]. Plants such as musli, pipal, palash, mulethi, amaltas, kesraj, and bottle gourd exhibit substantial lipid-lowering activities. Numerous studies have reported that the use of nutraceuticals and herbs like artichoke leaf extract, garlic, soluble fibers, nuts, and orange juice leads to a notable reduction in plasma lipid levels [7].

Belonging to the Asclepiadaceae family, Caralluma is commonly known as the milkweed family. While approximately 200 genera and 2500 species have been documented within the Asclepiadaceae family. Advanced genetic and molecular studies propose Asclepiadaceae as a subfamily in the Apocynaceae family [12]. Caralluma plants boast numerous medicinal properties, with applications in Arabic traditional herbs and Indian conventional medicines for managing cancer, tuberculosis, inflammation, skin problems, diabetes mellitus, and more. Pharmacological studies on Caralluma species have demonstrated anticancer [13], anti-eczemic, anti-inflammatory, antioxidant, antifungal, antidiabetic, and antimalarial activities [12].

Caralluma edulis, a medicinal plant, has garnered significant attention due to its therapeutic potential. Its traditional use in treating various ailments has spurred scientific investigations into its pharmacological properties. Among the numerous health benefits attributed

to *C. edulis*, its hypolipidemic and vasoprotective effects have garnered particular interest. This study aims to explore the hypolipidemic and vasoprotective potential of *C. edulis* through biochemical and histological analysis, offering promising avenues for the development of novel therapies for hyperlipidemia treatment.

2. MATERIALS AND METHODS

2.1. Chemicals

The chemicals of analytical grade were utilized in this study, including Atorvastatin (MediPak Pvt Ltd), Lipofundin (B. Braun), Fructose 25% w/v (Sigma Aldrich), Sodium Phenobarbital, Potassium chloride, Phenylephrine, Ketamine chloride and Acetylcholine, (Sigma Aldrich).

2.2. Equipment

The following equipment was employed: Tissue organ bath, Centrifuge machine Lab Chart 6.0 (AD instruments), Lyophilizer (Christ alpha 1-4 LD, Germany), Rotary evaporator (Stuart, Bibby Steriline Ltd. UK), and Power lab Data Acquisition System, Model No. ML865 (AD instruments).

2.3. Animal models

In this study we used local breed of rabbits of either sex (1-1.5 Kg) and male adult Wister Albino rats (150-200 g). All the animals were housed in the animal facility of the Pharmacology Department of College of Pharmacy, University of Sargodha. They were supplied with a regular pellet diet and tap water *ad libitum* and kept under standard environmental conditions (humidity; 50% ± 5%), (room temperature; 22 ± 2 ºC), and light and dark period of 12-hour each, following the guidelines of NIH (National Institute of Health) regarding the use and care of the animals. The experimental procedures and animal handling were carried out by following the rules and regulations of the National Research Council (NRC., 1996). The ethical approval for the use of laboratory animals were obtained by institutional animal ethical committee vide letter no 33637/AE.

2.4. Plant collection and identification

In this study, the succulent stems of *C. edulis* were utilized. Around 14 kg of *C. edulis* stems were gathered in November 2022 from the local market of District Malakand, KPK, Pakistan. Authentication was conducted by an Associate Professor, Dr. Hassan Sher, from Department of Plant Science, Swat University, Pakistan**.**

2.4.1. Preparation of plant extract

The hydro-methanolic extract of *C. edulis* or *C. edulis* methanolic extract was obtained using the cold maceration technique. First, the stems of the plant were collected, washed thoroughly to remove any dirt or impurities, and then shade dried. Once dried, they were powdered into coarse particles. Approximately 2 kg of the powdered material was weighed out and soaked in a mixture of 70% distilled water and 30% methanol, totaling 5 L, at room temperature for 3 days, with occasional shaking. After the maceration period, the material was filtered first through muslin cloth and then through Whatman No. 1 filter paper. The residue was subjected to another round of maceration for an additional 3 days, followed by filtration. The filtrate obtained was then processed using a rotary evaporator under controlled conditions: speed set at 55–60 rpm, temperature maintained at 35 ± 5 °C, and pressure adjusted to 45-50 Torr. This process yielded a semi-solid, thick paste-like extract, which was carefully removed from the rotary flask and weighed. The percentage yield was calculated to be 17%. Lastly, the extract was poured in a dry glass jar and stored at -8 °C in a refrigerator.

2.5. Experimental procedures

2.5.1. Lipofundin induced dyslipidemia

Initially, a Rabbit model was employed for the hypolipidemic study. The animals were randomly divided into five groups, each comprising six rabbits. Group-I was designated as the normal control. Group-II, the disease control, received intravenous lipofundin for eight consecutive days at a dose of 2 mg/Kg. Group-III served as the standard control, initially receiving intravenous lipofundin (2 mg/Kg) for eight days, followed by oral administration of atorvastatin (10 mg/Kg) for the subsequent 15 days. Similarly, Group-IV and Group-V

were experimental controls. They initially received intravenous lipofundin at a dose of 10 mg/Kg for eight consecutive days. Subsequently, they were orally administered with methanolic extract of *C. edulis* at doses of 250 mg/Kg and 500 mg/Kg, respectively, for the next eight consecutive days.

Upon completion of the experiment, the animals underwent overnight fasting. On the 24th day, we collected blood (approximately 3 mL) from the jugular vein of each rabbit. It was then centrifuged for ten minutes at 3000 rpm to get serum for the estimation of biochemical parameters. Then biochemical studies were performed on the serum to assess TG (Triglyceride), TC (Total Cholesterol) and HDL (High-Density Lipoprotein) and commercial kits of Human Germany were used for the analysis. The other parameters like AI (atherogenic index), CRI (Coronary Risk Index), LDL (Low Density Lipoprotein) and VLDL (Very Low Density Lipoprotein) levels were estimated from TG, TC and HDL values by using the following formulas [14]:

Estimation of VLDL = TG/5

Estimation of AI = TC-HDL/HDL

Estimation of LDL = TC-(HDL + VLDL)

Estimation of CRI = TC/HDL

2.5.2. Fructose induced hyperlipidemia

Adult male Wistar Albino rats weighing between 100-150g were randomly allocated into five groups, with six rats in each group. Rats in Group-I were fed a standard diet and served as the normal control. Group-II, designated as the disease control group, received oral administration of fructose (25% w/v) in their drinking water for 28 consecutive days to induce hyperlipidemia. Similarly, hyperlipidemia was induced in Groups III, IV, and V by orally administering fructose (25% w/v) in their drinking water for 14 consecutive days. Starting from the 15th day until the 28th day, Group-III was orally treated with 10 mg/Kg of atorvastatin two hours prior to fructose feeding. Likewise, Groups IV and V received oral doses of hydro-methanolic extract of *C. edulis* at doses of 250 mg/Kg and 500 mg/Kg, respectively, also two hours before fructose feeding from the 15th to the 28th day. After the completion of the experiment, the animals underwent overnight fasting. On the following day, blood samples were collected via cardiac puncture from each rat after euthanization. The blood samples were then centrifuged to obtain serum.

Then biochemical studies were performed on the serum to assess triglyceride (TG), Total cholesterol (TC) and high-density lipoprotein (HDL) and commercial kits of Human Germany were used for the analysis. The other parameters like AI (atherogenic index), CRI (coronary risk index), LDL (low density lipo protein) and VLDL (very low density lipoprotein) levels were estimated as indicated in section 2.5.1 [14]

2.5.3. Fructose-induced vascular dysfunction

In vitro vascular reactivity assessments were conducted on fructose-fed rats to evaluate endothelial integrity. The thoracic aorta of the rats was carefully dissected and cut into 3 mm long rings, with meticulous measures taken to prevent any damage to the endothelium. These rings were suspended in organ baths using two stainless steel wires inserted into the lumen between a clip and a force-displacement transducer. A resting tension of 2 g was applied to aid in achieving isometric force. The organ chamber was filled with 10 mL of oxygenated Krebs's Henseleit buffer solution (95% oxygen and 5% carbon dioxide), maintained at 37°C. The preparations were allowed to equilibrate with continuous exchange of Krebs's solution for approximately 1 hour. After the equilibration period, the ring segments were pre-contracted with KCl, and the active muscle tone of the ring segments was then contracted with 10-6 M phenylephrine. Upon reaching a stable contraction plateau, the relaxation response to cumulative acetylcholine concentrations (10^{-9} to 10^{-4} mole per liter) was measured [15].

2.6. Histopathological analysis of liver in fructose-fed rats (FFR)

The livers from both the normal control and treated rats were carefully extracted, washed with a normal saline

solution, and then fixed in a 10% buffered formalin solution for preservation. Subsequently, they underwent processing for histological studies. Histopathological examination was conducted on liver sections of 5-micron thickness, which were prepared by processing the liver tissues with paraffin and staining them with Hematoxylin and Eosin (H&E). The tissue samples were then examined under a light microscope to analyze the cellular structure and identify any pathological changes. Photomicrographs were captured using a Kodak digital 10-megapixel camera. Notable alterations in histopathological parameters, such as hepatic steatosis, areas of necrosis, vacuoles of varying sizes, and fatty degeneration, were observed in the liver sections of normal, control, and treated rats, enabling the assessment of significant structural changes in hepatic tissue architecture [16,17].

2.7. Free radical scavenging activity

The antioxidant capacity of the *C. edulis* extract was evaluated using the stable free radical scavenger DPPH. This involved assessing the rate of bleaching of the stable free radical at a specific absorption wavelength of 517 nm, where its absorption decreases upon reduction by a radical or antioxidant species. The methodology included preparing various concentrations of the hydro-methanolic extract of *C. edulis* (125-100 µg/mL) in methanol. Additionally, a 0.1 mM DPPH solution was prepared in methanol. Subsequently, 1 mL of the prepared DPPH solution was added to 3 mL of the different concentrations of plant extract solutions. The reaction mixture was then incubated for 30 minutes at room temperature (28 ± 2ºC). The absorbance of the reaction mixture was measured at a wavelength of 517 nm using a spectrophotometer. A control solution containing 100 µL of methanol in the DPPH solution was also prepared, and its absorbance was measured. The assay was conducted in triplicate. L-ascorbic acid was used as a reference. A higher radical scavenging capacity of the reaction mixture was indicated by a lower absorbance value [18]. The DPPH free radical scavenging capacity of the plant extract was calculated based on the ability to scavenge the DPPH radical by the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_{\text{Control}} - A_{\text{Sample}}/A_{\text{Control}}) \times 100]$$

Whereas:

A_{Control} is the absorbance of the control reaction; A Sample is the absorbance in the presence of a sample (plant extract).

2.8. Statistical analysis

The data were presented as means ± S.E.M. Statistical analysis was performed using GraphPad Prism 5. For all experiments, two-way ANOVA and Bonferroni's posttest were utilized. A significance level of $p < 0.05$ (95% confidence interval) was considered statistically significant.

3. RESULTS

3.1. Hypolipidemic activity of hydro-alcoholic extract of *C. edulis* in lipofundin-induced dyslipidemia

3.1.1. Effect on lipid profile

In the LIPO group, rats showed elevated levels of TC, TG, LDL, and VLDL. On the 23rd day, the LIPO group exhibited a significant increase ($P<0.05$) in TC (84.33 ± 4.25 mg/dL) and serum TG ($P<0.001$) to (112 ± 6.24 mg/dL) compared to the control group values (65 ± 2.8, 72.66 ± 4.33 mg/dL). The *C. edulis* hydro-methanolic extract demonstrated dose-dependent hypolipidemic effects. At a dosage of 500 mg/Kg, it notably attenuated the elevation of TC, TG, and LDL compared to the LIPO group on the 23rd day, while the reduction in HDL and VLDL was not statistically significant. *C. edulis* hydro-methanolic extract significantly ($P<0.001$) decreased serum TC to (59.33 ± 2.96 mg/dL) compared to the LIPO 23rd group (84.33 ± 4.25 mg/dL) and serum TG ($P<0.001$) to (81 ± 4.93 mg/dL) compared to the LIPO 23rd group (112 ± 6.24 mg/dL). Furthermore, it markedly reduced serum LDL levels to (12.80 ± 1.33 mg/dL) compared to the LIPO group on the 23rd day (29.93 ± 4.47 mg/dL), as illustrated in Table 1.

3.1.2. Effect on the atherogenic and coronary risk index

The 70% methanolic extract of *C. edulis* at a dosage of 500 mg/Kg exhibited a significant ($P<0.01$) reduction in

the values of the Atherogenic index (AI) compared to the LIPO 23rd day control group, from (1.63 ± 0.15 mg/dL) to (0.97 ± 0.11 mg/dL). Similarly, in the case of the coronary risk index (CRI), *C. edulis* at a dosage of 500 mg/Kg demonstrated a significant (P<0.01) decrease in CRI (1.97±0.11 mg/dL) compared to the diseased group (2.63±0.15 mg/dL), as outlined in Table 1.

Table 1: Effect of *C. edulis* extract on serum lipid profile, atherogenic (AI) and coronary risk index (CRI)

No	Parameter	Animal Groups					
		G-I (Control)	**G-II** (LIPO 8th day)	**G-II** (LIPO 23rd day)	**G-III** (ATOR)	**G-IV** (CE 250)	**G-V** (CE 500)
I	Serum TC (mg/dL)	65.00±2.8	86.6±4.40**	84.33±4.25*	61.00±4.93[b]	73.67±6.17	59.33±2.96[a]
II	Serum TG (mg/dL)	72.66±4.33	118.3±7.26***	112.0±6.24***	72.66±4.33[a]	96.00±12.42[c]	81.00±4.93[a]
III	HDL (mg/dL)	33.00±3.21	31.33±1.85	32.00±0.57	35.33±3.71	34.33±3.38	30.33±3.18
IV	VLDL (mg/dL)	14.53±0.86	23.66±1.45	22.4±1.24	14.53±0.86	19.20±2.48	16.2±0.98
V	LDL (mg/dL)	18.46±3.37	32.00±0.2	29.93±4.47	11.33±2.02[c]	20.13±3.57	12.80±1.33[c]
VI	AI (mg/dL)	1.007±0.24	1.76±0.03**	1.63.0±0.15*	0.73±0.06[a]	1.16±0.18	0.97±0.11[b]
VII	CRI (mg/dL)	2.007±0.24	2.76±0.03**	2.63±0.15*	1.73±0.06[a]	2.16±0.18	1.97±0.11 [b]

G-I= Normal Control, G-II = Disease Control (lipofundin 2 mg/Kg), G-III = Standard Control (Atorvastatin 10 mg/Kg), G-IV = *C. edulis* hydro-methanolic extract at dose 250 mg/Kg, G-V = *C. edulis* hydro-methanolic extract at dose 500 mg/Kg. Data are expressed as Mean ± SEM and two-way ANOVA was applied as statistical tool by using Graphpad Prism-7. The level of significance was denoted as: *P<0.05, **P<0.01, ***P<0.001 (compared with normal control). [a] P<0.001, [b] P<0.01, [c]P<0.05 (as compared to LIPO 23rd day control).

3.2. Antihyperlipidemic effect of hydro-methanolic extract of *C. edulis* in fructose-induced hyperlipidemia and vascular dysfunction

3.2.1. Effect on serum lipid profile

In the FFR group, rats exhibited increased levels of serum TC, TG, VLDL, and LDL. The FFR group demonstrated a significant (P<0.001) elevation in TC (106.66 ± 6.76 mg/dL) and TG (196.66 ± 7.26 mg/dL) compared to the control group (71 ± 2.30 mg/dL, 91.66 ± 6.00 mg/dL) for TC and TG, respectively. *C. edulis* hydro-methanolic extract demonstrated dose-dependent hypolipidemic effects. At a dosage of 500 mg/Kg, it significantly attenuated the rise in serum TC, TG, and VLDL compared to the FFR group, while the reduction in HDL and LDL was not significant. *C. edulis* at 500 mg/Kg significantly (P<0.001) reduced serum TC and TG to (73.33 ± 3.52 mg/dL) and (95.66 ± 7.21 mg/dL), respectively, in contrast to the FFR group (106.66 ± 6.76 mg/dL, 196.66 ± 7.26 mg/dL). Furthermore, FFR + CE 500 (*C. edulis* 500 mg/Kg) also significantly (p<0.01) decreased serum VLDL (19.13 ± 1.44 mg/dl) compared to the FFR group (39.33 ± 1.45 mg/dL). Additionally, FFR+

ATOR group significantly (p<0.001) decreased TC, TG, and VLDL levels (63.33 ± 4.63 mg/dL, 72.66 ± 4.33 mg/dL, 14.53 ± 0.86 mg/dL) compared to the FFR group (106.66 ± 6.76 mg/dL, 196.66 ± 7.26 mg/dL, 39.33 ± 1.45 mg/dL), respectively (Table 2).

3.2.2. Effect on Atherogenic (AI) and Coronary risk index (CRI)

The CE 500 group (*C. edulis* hydro-methanolic extract at dose 500 mg/Kg), demonstrated a significant (p<0.01) decrease in the Atherogenic index (0.84 ± 0.125 mg/dL) compared to the FFR (Fructose Fed Rats) group (1.43 ± 0.127 mg/dL). Moreover, the FFR + CE 500 group (group administered with fructose 25% w/v and *C. edulis* hydro-methanolic extract at dose 500 mg/Kg) also exhibited a significant (p<0.01) decrease in Coronary risk index (1.84 ± 0.125 mg/dL) compared to the FFR group (2.43 ± 0.127 mg/dL), as depicted in Table 2.

Table 2: Effect of hydro-methanolic extract of *C. edulis* on serum lipid profile, atherogenic (AI) and coronary risk index (CRI)

No.	Parameter	Animal Groups				
		G-I (Normal Control)	G-II (FFR)	G-III FFR+Ator	G-IV FFR+CE 250	G-V FFR+CE 500
I	Serum TC	71.0±2.30	106.66± 6.76***	63.33±4.63[a]	89±4.35[c]	73.33±3.52[a]
II	Serum TG	91.66±6.00	196.66±7.26***	72.66±4.33[a]	111.66±4.40[a]	95.66±7.21[a]
III	HDL	36.66±2.40	44.33±5.36	33.33±4.91	44.00±3.05	40.00±2.88
IV	VLDL	18.33±1.20	39.33±1.45**	14.53±0.86[a]	22.33±0.88[c]	19.13±1.44[b]
V	LDL	16.00±0.57	23.0±2.88	15.46±2.14	22.66±2.02	14.2±2.69
VI	AI	0.97±0.066	1.43±0.127*	0.93±0.14[c]	1.03±0.08[c]	0.84±0.125[b]
VII	CRI	1.97±0.06	2.43±0.127*	1.93±0.14[c]	2.03±0.08 [c]	1.84±0.125[b]

G-I= Normal Control, G-II = Disease Control (fructose (25% w/v)), G-III = Standard Control (fructose 25% w/v+Atorvastatin 10 mg/Kg), G-IV = fructose 25% w/v+ *C. edulis* hydro-methanolic extract at dose 250 mg/Kg, G-V = fructose 25% w/v+ *C. edulis* hydro-methanolic extract at dose 500 mg/Kg. Data are expressed as Mean ± SEM and two-way ANOVA was applied as statistical tool by using Graphpad Prism-7. The level of significance was denoted as: *P<0.05, **P<0.01, ***P<0.001 (compared with normal control). [a] P<0.001, [b] P<0.01, [c]P<0.05 (as compared to Fructose control). FFR stands for fructose fed rat and CE stands for *C. edulis*, hydro-methanolic extract.

3.3. The vasorelaxant effect of *C. edulis* extract on fructose-induced vascular dysfunction

Ach (10^{-9} to 10^{-4} M) elicited concentration-dependent relaxation in pre-constricted abdominal aorta rings treated with 10^{-6} M phenylephrine. Fructose-fed rats displayed a significant (P<0.001) impairment in maximum endothelium-dependent vascular relaxation compared to the Control group. Hydro-methanolic extract of *C. edulis* demonstrated a dose-dependent effect on endothelium-dependent vasorelaxation. Both CE 500 mg/Kg and atorvastatin effectively mitigated the impairment of vascular endothelial function induced by fructose administration. Notably, the highest dose of the plant extract (CE 500 mg/Kg) exhibited superior efficacy in preserving vascular endothelial integrity (Fig. 1).

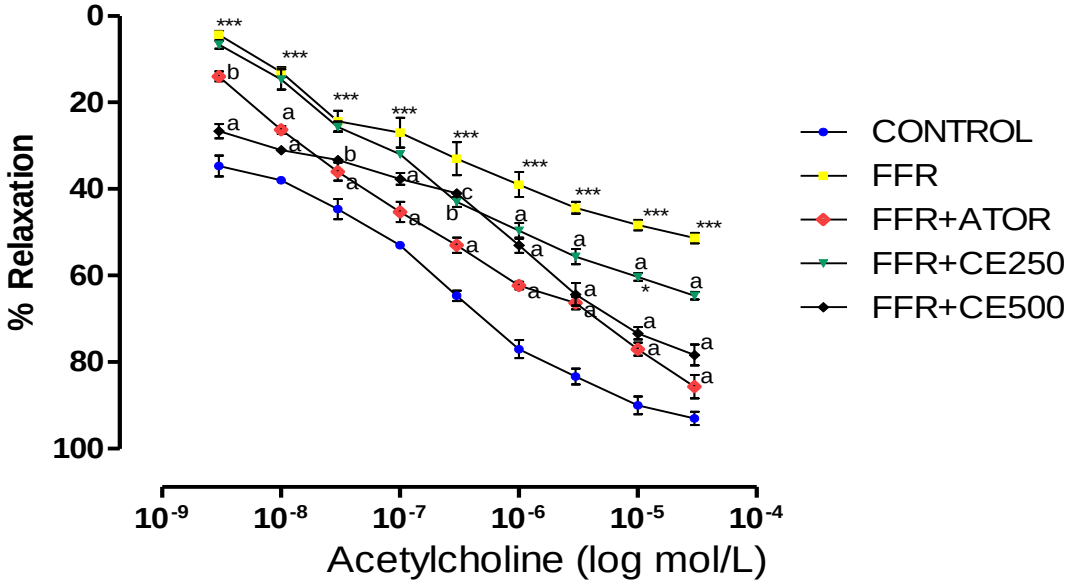

Fig 1: Endothelium-dependent vascular relaxations by Ach in rat thoracic aorta rings.

Data are expressed as Mean ± SEM and two-way ANOVA was applied as statistical tool by using Graphpad Prism-7. The level of significance was denoted as: *P<0.05, **P<0.01, ***P<0.001 (compared with normal control). [a] P<0.001, [b] P<0.01, [c] P<0.05 (as compared to Fructose control). FFR stands for fructose fed rat and CE stands for *C. edulis*, hydro-methanolic extract.

3.4. Hepatoprotective activity of hydro-methanolic extract of *C. edulis* in fructose-fed rats

Histopathological examination of normal rat liver sections revealed an intact hepatic architecture. Conversely, liver sections from fructose-fed rats exhibited significant alterations, including focal necrosis, steatosis, and congestion of the central hepatic vein. Treatment with the highest dose of CE (500 mg/Kg) markedly ameliorated these histopathological changes compared to the lower dose of CE (250 mg/Kg), which still exhibited signs of steatosis and hepatic architectural damage. Rats treated with *C. edulis* hydro-methanolic extract at 500 mg/Kg, as well as those treated with atorvastatin, demonstrated hepatoprotective effects, characterized by reduced hepatocellular necrosis, diminished fatty degeneration, and decreased central vein congestion, in contrast to fructose-fed rats (Fig.2).

3.5. The Antioxidant potential of *C. edulis* extract by DPPH scavenging capacity

The increase in the DPPH scavenging capacity of *C. edulis* was observed in a dependent manner (Table 3).

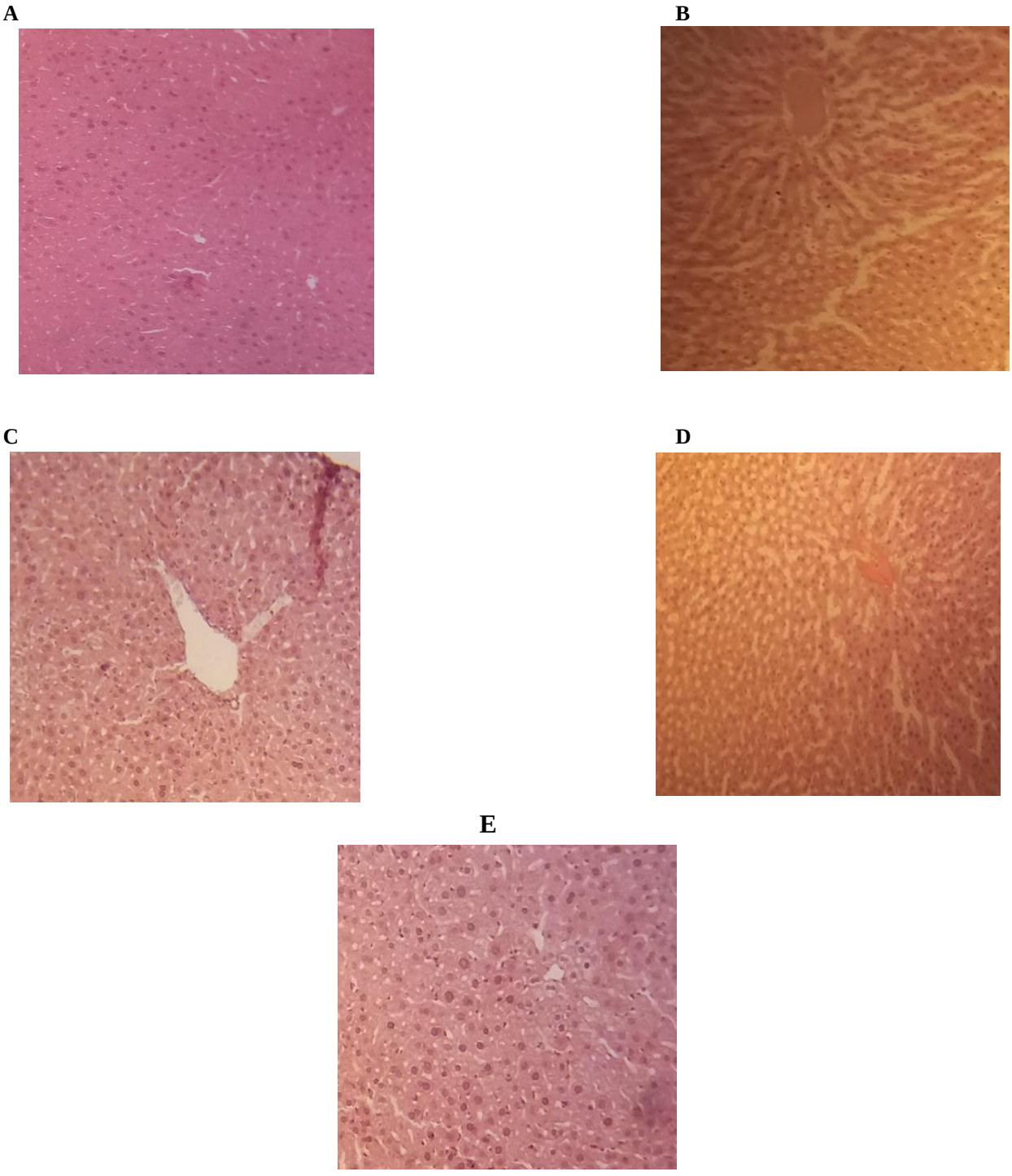

Fig 2: Histopathological examination of rat liver sections

(H and E x 200); A = Normal Group; B = Fructose fed group; C = Standard group; D = CE 250 mg/Kg; E = CE 500 mg/Kg.

Table 3: The antioxidant potential of *C. edulis*

Concentration (ug/mL)	% Antioxidant potential	
	Ascorbic acid	CE
125	80.74±0.026	32.14±0.007***
250	83.45±0.018	40.43±0.018***
500	86.64±0.012	50.45±0.020***
10	88.53±0.023	64.24±0.026***

Values were expressed as Mean ± SEM. Data were evaluated by means of two-way ANOVA. *P<0.05, **P<0.01, ***P<0.001

DISCUSSION

The condition of hyperlipidemia, characterized by elevated plasma lipid levels and altered lipoprotein profiles, significantly contributes to the development of atherosclerosis and cardiovascular diseases [19]. The World Health Organization (WHO) has projected a substantial increase in cardiovascular-related deaths globally, underscoring the urgency of addressing this health concern [20]. Hyperlipidemia often arises from dietary factors, sedentary lifestyles, and genetic predispositions, among other factors, and is exacerbated by various diseases such as diabetes and hypothyroidism [19]. Liver enzymes, particularly 3-hydroxy-3-methylglutaryl (HMG-CoA reductase), play a crucial role in cholesterol synthesis, making them key targets for therapeutic intervention [5]. Currently, anti-hyperlipidemic treatment primarily involves the use of various pharmaceutical agents such as HMG-Co-A reductase inhibitors (statins), fibrates, cholesterol absorption inhibitors, and bile acid sequestrants. While these medications effectively target cholesterol synthesis and lipid metabolism, they often come with cost-related challenges and significant long-term side effects. Consequently, there has been a growing interest in alternative therapies, particularly herbal medicines, which offer a cost-effective and safer approach with lower toxicity compared to synthetic drugs. The World Health Organization (WHO) has also advocated for the use of herbal medicine as an alternative therapy in developing countries [21].

In this study, we utilized the hydro-methanolic extract of *C. edulis* stems to evaluate its anti-hyperlipidemic properties in models of hyperlipidemia induced by lipofundin and fructose, as well as vascular dysfunction in rabbits and rats. Administration of lipofundin 20% for eight consecutive days led to elevated serum levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL). Lipofundin, containing long-chain triglycerides derived from soybean oil, has previously been associated with fat accumulation and alterations in aortic architecture, contributing to the development of atherosclerotic lesions in rabbits. These effects may be attributed to the generation of systemic lipid peroxidation (LPO) products, leading to oxidative stress and depletion of antioxidants [22]. The *C. edulis* hydro-methanolic extract demonstrated significant reductions in serum cholesterol, triglycerides, and LDL levels in rabbits, comparable to the effects of atorvastatin administered at a dose of 500 mg/Kg orally. This hypocholesterolemic activity of the plant extract may be attributed to the inactivation of the HMG-Co-A reductase enzyme, potentially mediated by the phosphorylation of protein kinase (PKA) [23].

Our experimental plant extract, *C. edulis*, contains abundant glycosides, primarily flavone and megastigmane glycosides [12]. The lipid-lowering potential of this extract may be attributed to the presence of polyphenols, which are known for their ability to limit the incidence of atherosclerosis and other cardiovascular diseases by neutralizing oxidizing free radicals [24]. Previous studies have also reported that polyphenolic compounds, such as

flavonoids, exhibit strong antioxidant potential [25]. To assess the antioxidant capacity of our plant extract, we performed a DPPH radical scavenging assay. The hydro-methanolic extract of *C. edulis* exhibited robust antioxidant activity, which may contribute to the neutralization of oxidant species causing oxidative damage to lipid membranes and the reduction of peroxide radicals through its glycoprotein scavenging activity [23].

Various research findings suggest several mechanisms of fructose-induced hyperlipidemia. Previous studies have indicated that fructose alters enzyme activities that regulate hepatic carbohydrate metabolism, thereby impacting lipid metabolism and leading to hepatic insulin resistance [26]. In our study, we utilized a 25% w/v fructose solution in drinking water for 28 days to induce hyperlipidemia and vascular dysfunction. Fructose administration resulted in elevated levels of serum total cholesterol (TC), triglycerides (TG), very low-density lipoproteins (VLDL), and low-density lipoproteins (LDL), along with a significant impairment in endothelium-dependent vascular relaxation [27]. These findings align with previous research indicating that fructose administration is associated with insulin resistance, hypertriglyceridemia, hypercholesterolemia, and vascular dysfunction. Additionally, other studies have reported that fructose intake leads to impaired endothelial vascular relaxations, with insulin resistance contributing to compromised endothelial function [28]. This aligns with research indicating that the conversion of fructose carbon atoms into glycerol 3-phosphate may contribute to increased triglyceride levels in fructose-fed rats [26]. Our study found that the hydro-methanolic extract of *C. edulis* exhibited strong therapeutic potential against hyperlipidemia at a dose of 500 mg/Kg. This extract significantly reduced serum cholesterol, triglycerides, and LDL levels in fructose-fed rats. Elevated levels of cholesterol, triglycerides, LDL, and low levels of HDL are associated with the development of cardiovascular diseases. The plant may exert its antihyperlipidemic activity by modulating phosphofructokinase enzyme activity, which is crucial for lipogenesis [29]. The significant reduction in triglyceride levels observed with the hydro-methanolic extract of *C. edulis* suggests a potential mechanism involving the inhibition of chylomicron-triglyceride complex discharge into the lymph by the plant extract. Additionally, the study suggests that the plant's anti-hyperlipidemic activity could be associated with increased lecithin acyl transferase activity on HDL [30]. It's likely that the hydro-methanolic extract of *C. edulis* contains saponins, tannins, and triterpenoids, similar to other species of the Caralluma genus like *Caralluma tubercala*, which possess saponins[31]. Studies indicate that saponins in plant extracts form complexes with cholesterol, thereby modifying cholesterol metabolism. Furthermore, the presence of saponins, tannins, and triterpenoids in plant extracts is associated with hypolipidemic activity, suggesting that the reduction in serum lipid levels observed with *C. edulis* may be attributed to these active phytoconstituents, as observed in other species like *Caralluma adcendens* and *Caralluma fimbriata* [32].

Previous research indicates that endothelial damage plays a crucial role in the progression of vascular diseases [33]. Hyperlipidemia often contributes to endothelial impairment, leading to reduced availability of nitric oxide (NO) and consequent vascular dysfunction. The hydro-methanolic extract of *C. edulis* significantly mitigated vascular damage induced by fructose administration and demonstrated a dose-dependent improvement in endothelium-dependent vascular relaxation in response to Ach (10^{-9} to 10^{-4} M) in pre-constricted abdominal aorta rings with 10^{-6} M phenylephrine, particularly at the highest dose (500 mg/Kg), yielding results comparable to atorvastatin. The restoration of vascular endothelial damage by the plant extract may be attributed to enhanced NO availability and inhibition of endothelial nitric oxide synthase (eNOS) activity [34].

The effectiveness of *C. edulis* against hypertension, atherosclerosis, obesity and hyperlipidemia has also been

scientifically proved. But the current study conducted on the same plant is different in the sense that two animal models; rat model and rabbit model, have been used here. Furthermore, the detailed vasoprotective effects of the *C. edulis* have been reported in this study [35-36]. The previous studies have indicated that high levels of fructose (25% w/v) can lead to increased liver weight in animals. Administration of high doses of fructose to the liver may result in rapid lipogenesis, accumulation of triglycerides, and fat in hepatocytes [5,37]. In our study, histopathological examinations of isolated rat liver sections were conducted to evaluate the hepatoprotective effects of the plant. These examinations revealed that fructose administration caused structural damage to hepatocytes, potentially associated with steatosis or increased hepatic cholesterol synthesis due to elevated hepatic HMG Co-A reductase activity [29]. However, the hydro-methanolic extract of *C. edulis* demonstrated hepatoprotective activity, significantly improving hepatic architecture when administered at a dose of 500 mg/Kg/p.o.

Hyperlipidemia poses a significant risk factor for the development of atherosclerosis, which can lead to coronary heart disease and other vascular conditions [23]. Atherogenic and coronary risk indexes are crucial determinants in assessing the risk of atherosclerotic plaque formation. The atherogenic index reflects the deposition of fatty substances and lipids in arterial walls, impacting vital organs such as the aorta, liver, and kidneys. Higher values of the atherogenic index indicate an increased risk of damage to these organs [5,38,39]. In our study, the hydro-methanolic extract of *C. edulis* (administered at 500 mg/Kg/p.o) significantly reduced both atherogenic and coronary risk indexes ($P<0.01$) compared to both lipofundin-administered rabbits and fructose-fed rats. This substantial reduction suggests that certain pharmacologically active components present in *C. edulis* may contribute to its therapeutic potential in preventing atherosclerosis.

CONCLUSIONS

The findings indicate that the hydro-methanolic extract of *C. edulis* exhibits dose-dependent hypolipidemic activity, as well as antioxidant and vasoprotective properties. This suggests that specific active constituents within the plant extract may be responsible for its lipid-lowering effects. Further research is needed to fractionate the extract based on its activity, which would facilitate the identification and characterization of these constituents. Understanding their precise mode of action in experimental models could provide valuable insights into the therapeutic potential of *C. edulis*.

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COMPETING INTERESTS

No competing interests were declared by authors.

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نقص شحميات الدم وإمكانية حماية الأوعية الدموية في *Caralluma edulis* دراسة نسيجية وكيميائية حيوية

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ملخص

(Apocynaceae**)** معروف جيدًا بخصائصه الطبية، بما في ذلك الأنشطة المضادة للأكسدة والالتهابات والمضادة للميكروبات وخافضة السكر في الدم، وقد تم استخدامه كعلاج قيم في مختلف الثقافات. يهدف هذا العمل العلمي إلى التحقق من فعالية C. edulis في خفض مستوى الدهون باستخدام نموذجين حيوانيين لارتفاع نسبة الدهون في الدم: الأرانب التي يسببها الليبوفوندين والفئران التي يسببها الفركتوز. تم إعطاء الليبوفوندين عن طريق الوريد بمعدل 2 مل / كجم لمدة 23 يومًا، في حين تم إعطاء الفركتوز (25٪ وزن / حجم) لمدة 28 يومًا متتالية عن طريق إذابته في مياه الشرب لتحفيز اضطراب شحوم الدم واختلال وظائف الأوعية الدموية. تم إعطاء المستخلص المائي الكحولي لـ C. edulis عن طريق الفم (250 و 500 مجم / كجم) للمجموعات التجريبية، بينما تم إعطاء أتورفاستاتين (10 مجم / كجم ص) لحيوانات المجموعة الضابطة القياسية فقط. تم جمع عينات الدم لتقييم المعايير البيوكيميائية المختلفة. علاوة على ذلك، تم إجراء الفحص النسيجي لأنسجة الكبد والشريان الأورطي الصدري من الجرذان التي تتغذى على الفركتوز، إلى جانب تقييم خصائصها المرخية للأوعية الدموية. أظهر المستخلص المائي الميثانولي لـ C. edulis تأثيرات خافضة للدهون تعتمد على الجرعة، مما يقلل بشكل كبير من نسبة الكوليسترول في الدم والدهون الثلاثية والبروتينات الدهنية منخفضة الكثافة بجرعة 500 ملغم / كغم في كلا النموذجين، مقارنة بأتورفاستاتين. علاوة على ذلك، أظهر المستخلص المائي الكحولي نشاطًا كبيرًا يعتمد على ارتخاء الأوعية الدموية وتأثيرات وقائية للكبد في الجرذان التي تتغذى على الفركتوز. أظهر C. edulis أيضًا إمكانات مضادة للأكسدة من خلال نشاط مسح الجذور الحرة. تشير هذه النتائج إلى أن C. edulis يمتلك خصائص خافضة لشحوم الدم وواقية للأوعية الدموية، والتي تعزى على الأرجح إلى مكونات دوائية نشطة محددة، مما يدعم استخدامه التقليدي.

الكلمات الدالة: ليبوفوندين، فرط شحميات الدم الناجم عن الفركتوز، أتورفاستاتين، دراسات مرخيات الأوعية، الكولسترول في الدم.

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A Comprehensive Review of *Canarium odontophyllum* Fruits and Their Multifaceted Benefits

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ABSTRACT

The rare plant *Canarium odontophyllum* Miq., locally known as "dabai," is found in Sarawak, Malaysia. The fruit is often consumed for its purported health benefits, but it remains underutilized due to its short shelf life, particularly in rural areas. Despite being a seasonal fruit, dabai is recognized for its nutritional value, and some studies have explored *Canarium odontophyllum*'s biological properties to support its use as a nutraceutical and health supplement. The pulp, peel, and kernel of the dabai fruit are all nutrient-dense, bioactive components, rich in dietary fiber. The fruit contains flavonoids, tannins, and terpenoids. Furthermore, crude extracts from *Canarium odontophyllum* have shown a variety of therapeutic benefits, including antimicrobial, antimalarial, antioxidant, anticancer, antidiabetic, and antifungal effects. Dabai is particularly notable for its high nutritional content, which includes significant amounts of protein, fat, and carbohydrates. Electronic databases such as PubMed, Scopus, Web of Science, and Google Scholar were searched from 1980 through November 2023 to identify relevant publications.

Keywords: *Canarium odontophyllum*; dabai; physical properties; phytoconstituents; nutritional values; pharmacological activity.

1. INTRODUCTION

In recent times, nutraceuticals and functional foods have emerged as a new frontier in natural therapeutic and protective agents. They have emerged as a safer alternative to medicinal herbs. This is because nutraceuticals are derived from plant foods that are regularly and continually consumed by humans without adverse health effects [1]. In contrast, many medicinal herbs are not commonly part of the regular diets, thereby nutraceuticals are considered safer. Nutraceuticals, derived from botanical or animal food sources, are bioactive constituents used to prevent or treat chronic diseases and are available in pharmaceutical forms. Functional foods, rich in bioactive constituents, protect against chronic diseases and are consumed in their standard food product form. Both nutraceuticals and functional foods can act as protective agents against diseases or complement drugs, allowing for a reduction in drug dose and its adverse effects [2]. The phytoconstituents of plants are used as antioxidants, antibacterial, anthelmintic, blood coagulants, diuretics, larvicides, and laxatives [3-7].

Borneo's native dabai (*Canarium odontophyllum* Miq.) is a member of the Burseraceae family of fruit-bearing plants. The fruit's rich nutritional content, which promotes health, makes it a delicacy in Sarawak, where residents eat it as a snack. *C. odontophyllum* fruit is considered an underutilized fruit because, despite its abundance, its

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potential is still mostly unrealized [8]. The Malaysian Agricultural Research and Development Institute has introduced dabai pickles and frozen dabai pulp to assure a continuous supply even during the off seasons, thereby expediting the growth of the dabai market. The produced products improve dabai fruit's potential for further exploration and allow for consistent processing of the fruit throughout the year.

Recent attention has been given to the nutritional values and health benefits of *C. odontophyllum*, with increasing recognition and thorough investigation. The current trend in literature focuses on profiling the phytochemical content and biological activities of various parts of the *C. odontophyllum*, including the skin, flesh or pulp, kernel, and leaf. The versatility of *C. odontophyllum* fruit (Figure 1) allows for its transformation into various food products. Presently, several food items incorporate dabai, such as dabai fried rice, dabai sauce, dabai mayonnaise, dabai ice cream, and dried dabai [9]. With the advancements in food research and development technology, there is potential for the creation of more dabai-based products. Leveraging the complete utilization of dabai fruit presents opportunities for profitable goods in the food industry and other sectors, offering a diverse range of food-based and waste-processing products with significant economic importance.

Figure 1: *Canarium odontophyllum* Miq. (Dabai) fruits [Adopted from Ref. 10]

2. METHOD

In this paper, electronic databases such as PubMed, Scopus, Web of Science, and Google Scholar, were searched from the start of 1980 to the end of November 2023 to extract pertinent publications. The terms used for search were: "*Canarium odontophyllum*" and "phytoconstituents" or "lung" or "biological activity" or "pharmacological activity" "nutraceuticals" or "propagation" or "culinary use" or "traditional use".

3. *CANARIUM ODONTOPHYLLUM* Miq. (DABAI)

3.1. Origin

Sarawak, a region in Malaysia, is renowned for its rich diversity of plant species, particularly indigenous fruits and vegetables, which play a crucial role in the diets of rural communities. Despite the growing popularity of these indigenous fruits among a significant portion of the population, many of these fruits remain underutilized, not reaching their full potential. Approximately 100 species of fruit-bearing trees of the Burseraceae family make up the large genus Canarium. These trees are primarily found in tropical Asia and the Pacific, as well as Africa [11]. More than half of Canarium spp. is native to the old-world tropics of southeast Asia [12]. Within this genus, two species have received notable attention in research, which are the African black olive (*C. sweinfurthii*) and the Chinese olive (*C. album*) [13]. In the context of Malaysia, four distinct Canarium species have been identified. Among these, *C. odontophyllum* stands out as the most popular and well-recognized species [14]. This highlights the significance and popularity of *C. odontophyllum*' within Malaysia and underscores its potential for various applications and commercialization within the region.

C. odontophyllum is a popular indigenous fruit in Sarawak with the potential to become a specialty fruit for the region. Its resemblance to olives in appearance, smooth texture, and rich flavor has earned it the nickname 'Sibu olive.' Belonging to the Burseraceae family of the

Sapindales order within the Eudicotyledoneae class, this fruit is part of a family comprising 16 genera and approximately 550 species found in tropical regions across the globe [15]. Despite its promising attributes, *C. odontophyllum* remains relatively underexplored due to a lack of promotion. *C. odontophyllum* is a seasonal fruit and is abundant in Sibu, a town in Sarawak, Malaysia. It ranks among the top six highly prized indigenous fruits in Sarawak and holds promise for commercialization. This fruit has two peak production seasons, typically occurring in July-August and November-December, subject to weather patterns [16].

Within the local markets, various genotypes of *C. odontophyllum* fruit could be found, with the six most common ones being Besar, Biasa, Jernah, Bujur, Seluang, and Bulat (Figure 2). These different genotypes exhibit variations in physical properties, sensory characteristics, and nutritional values [17]. Chew et al. (2011) reported that the nutritional and physical features of fruits are affected by cultivar, growing region, climate, maturity, and cultural techniques [13]. Determining the physical attributes and nutritional worth of *C. odontophyllum* fruit from various genotypes would therefore aid in the selection of better plants that bear fruits with desired qualities aimed at particular food and medicinal markets.

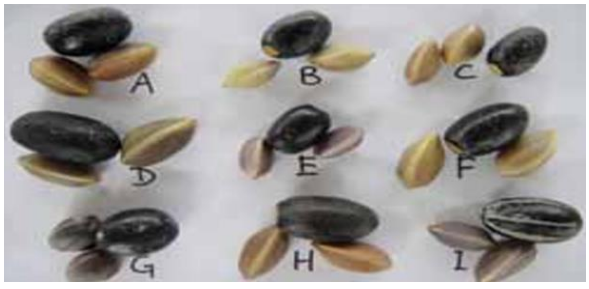

Figure 2: **Different genotypes of *C. odontophyllum* fruit: Besar (A), Biasa (B), Jernah (C), Bujur (D), Seluang (E) and Bulat (G)** [Adopted from Ref. 18]

3.2. *C. odontophyllum* propagation

The *Canarium odontophyllum*, or *C. odontophyllum* tree, is naturally found in specific regions of Sarawak, particularly along riverbanks in areas such as Sibu, Sarikei, Kapit, and Limbang Divisions, except in swamps and coastal sands. While *C. odontophyllum* trees grow naturally in the forests of Sarawak, there is a growing trend of cultivating them in regions like Sarikei, Sibu, and Kapit Divisions. *C. odontophyllum* fruits are seldom marketed beyond their grown area because the supply is still low and easily damaged by heat. Given the economic potential and value of *C. odontophyllum* as an indigenous fruit in Sarawak, there is a growing need for research to facilitate mass propagation of *C. odontophyllum* trees.

The uncommon tropical fruit *C. odontophyllum* is mostly propagated from seeds. The inconsistent and poor germination rate of this fruit species' seeds is shown by the paucity of documentation available on the subject [18]. It is crucial to acquire improved understanding and techniques for handling the seeds as planting materials because fruit supply is seasonal. This will make it possible to cultivate *C. odontophyllum* trees more effectively, guaranteeing a steady supply of fruit to satisfy consumer demand. Efforts to improve the propagation and cultivation of *C. odontophyllum* trees can not only benefit the local economy but also promote the broader appreciation and commercialization of this unique and culturally significant fruit in Sarawak.

It is better to sow *C. odontphyllum* seeds fresh. A study conducted in 2020 by Masarip et al. found that seeds taken from fresh fruits could withstand rapid desiccation utilizing desiccation bags (DBs) to moisture content (MC) of less than 10% [19]. The disadvantage of this rapid desiccation technique is that it may cause tiny fissures in the endocarp, which is not good for storing seeds. However, this quick desiccation method has a drawback as it can result in fine cracks in the endocarp, which is not ideal for seed storage. On the other hand, a slower desiccation process to achieve moisture content below

10% at a temperature of 40°C in a convection oven was found to be detrimental to the seeds. This means that attempting to dry the seeds slowly at these conditions may harm their viability. Maintaining low seed moisture content, especially below 10%, is crucial for successful seed storage and preventing microbial damage. Unfortunately, this poses a challenge when propagating *C. odontophyllum* through seeds. As a result, alternative methods, such as vegetative propagation, may be more reliable for cultivating *C. odontophyllum* trees, particularly when dealing with seeds that may not meet the required quality for germination.

To propagate *C. odontophyllum* trees, both traditional vegetative propagation methods and advanced in vitro techniques can be employed to produce planting materials. Conventional vegetative propagation, such as bud grafting, is a widely used method. It involves taking buds from hermaphrodite *C. odontophyllum* trees and grafting them onto seedling rootstocks. These grafted buds will develop into plants that are genetically identical to the parent *C. odontophyllum* tree and bear fruit even earlier than those propagated by seeds. Nevertheless, the utilization of bud grafting for *C. odontophyllum* propagation is hindered by the challenges associated with bud takes and bud break. Bud take refers to the successful union between a bud and a rootstock, while bud break is the process in which the bud elongates. An alternate approach is in vitro cultivation, which has the capacity to efficiently grow genetically identical plants on a large scale. Nevertheless, there has been no prior endeavor to employ this method for *C. odontophyllum* transmission, resulting in a dearth of information regarding the optimal cultural conditions and medium.

To ensure the production of fruit, it is highly suggested to use bud-grafted hermaphrodite plants. As a consequence of this method, *C. odontophyllum* trees exhibit precocious fruiting, commencing as early as 3 to 5 years post-planting. The Agriculture Research Centre in Semongok, Sarawak, Malaysia, has identified two exceptional bud-grafted clones, specifically 'Laja' and 'Lulong,' for the purpose of commercial production. At the onset of the reproductive phase, a tree that has been grafted with a bud can produce approximately 10 kg of fruit. Over time, as the tree grows and becomes at least 10 years old, this output steadily rises to 80-100 kg per tree. This method presents significant economic opportunities, as each individual tree has the ability to generate a minimum gross annual return of MYR 9,000.00. Furthermore, a hectare of land, with trees planted at a spacing of 12 m x 12 m, can yield a minimum gross annual return of MYR 612,000.00. Utilizing bud-grafted hermaphrodite plants for *C. odontophyllum* cultivation offers substantial benefits [20].

3.3. Physiology and characteristics

The tall, straight trunk of the *C. odontophyllum* tree (Figure 3), which normally begins to branch at a height of roughly 2-3 m above the ground, is what makes the tree distinctive. It can reach a height of over 20 m and a stem girth of more than 150 cm. The tree has a round, dense crown and gray-brown bark. With a terminal leaflet, the spirally arranged leaves have three to eight pairs of pinnately arranged leaflets. These leaflets have an oblong to lanceolate shape and measure between 4 and 11 cm in width and 9.5 to 28 cm in length.

Figure 3: Female *C. odontophyllum* tree that is 15 years old with a height of about 20 m
[Adopted from Ref. 21]

C. odontophyllum trees possess androdiecious traits, wherein they bear staminate (male) and hermaphrodite (possessing both male and female components) inflorescences on separate plants. Nevertheless, instances of flowers with prominent stamens and underdeveloped pistils have also been documented. Staminate plants exhibit elongated and slender inflorescences containing diminutive flowers, whereas hermaphrodite plants possess compact and stout inflorescences with significantly bigger blooms. The hermaphrodite blooms are situated on terminal inflorescences. Male trees generally initiate the process of flowering at a relatively young age, normally around 4-5 years. In contrast, hermaphrodite trees commence flowering and producing fruit at a later stage, approximately 6-8 years after being planted. Juvenile *C. odontophyllum* trees have the capacity to produce up to 10 kg of *C. odontophyllum* fruit, whilst fully grown trees (aged 10 years or more) can generate a range of 80 to 100 kg [22]. This suggests that during each season, an estimated 200,000 to 500,000 kg of *C. odontophyllum* fruit could be harvested. *C. odontophyllum* trees have the potential to reach heights of 30 to 40 m and can live for approximately 40 years [23]. Fruit production typically begins around 5 years after planting, and a fully mature tree can yield up to 800 kg of fruit all at once [24].

The *C. odontophyllum* tree belongs to the *Canarium L.* species, which has the natural ability to flourish in various soil types. The optimal circumstances for the growth of these trees are characterized by a moist, deep, crumbly, and organically rich sandy loam soil with a pH level ranging from 4.5 to 6.5. In addition, they have the ability to withstand mildly alkaline conditions with a pH of up to 7.4 and can adjust to poorly drain woodland habitats [25]. A recommended spacing of 10 meters is suggested between *C. odontophyllum* trees, which mean that approximately 100 trees can be planted in one hectare of orchard land [22].

The *C. odontophyllum* fruit consists of three primary components: an exterior skin known as the epidermis, a fleshy interior called the mesocarp, and a solid seed referred to as the endocarp. The composition of this fruit typically consists of approximately 5.6% skin, 61.4% flesh, and 37% seed. This categorizes it as a drupe berry, characterized by a thin skin that encloses the flesh and seed [26]. The endocarp, the innermost layer, is characterized by its durability and thickness, measuring around 2.5 to 3.5 cm in length and 1.6 to 2.0 cm in diameter [27]. The *C. odontophyllum* fruit's flesh exhibits varying thickness, ranging from 0.2 to 0.7 cm, and its hue spans from pale yellow to golden. The seed contained within the fruit exhibits a sub-triangular morphology, characterized by three distinct chambers (Figure 4). The *C. odontophyllum* seed possesses a resilient and lignified endocarp that envelops a consumable cotyledon internally, and the morphological characteristics of this shell closely match those of a palm kernel shell [16].

Figure 4: **Seed and kernel of *C. odontophyllum* fruits** [Adopted from Ref. 23]

The *C. odontophyllum* fruit is distinguished by its elongated form, delicate and consumable outer layer, and its inner meat, which can be either white or yellow, with differences observed among different cultivars. It has a distinctive taste [28]. The immature *C. odontophyllum* fruits are white (Figure 5). When *C. odontophyllum* fruit is fully ripe, its skin takes on a color between black and blue (Figure 5), mainly due to the presence of the pigment anthocyanin

(cyanine-3-glucoside) in the skin [29]. The fruit is usually oval or ellipsoid in shape, with a length of approximately 3.5 to 4.0 cm and a width of 2.0 to 2.5 cm. The weight of a single *C. odontophyllum* fruit can reach up to 18 grams, and the fruit is composed of pulp, peel, and kernel [30]. These discoveries are consistent with previous research conducted by Prasad and colleagues in 2010, which also reported that *C. odontophyllum* fruit has an elongated shape, with a length ranging from 3 to 4 centimeters and a weight between 10 to 13 grams per fruit [31].

Figure 5: **Immature *C. odontophyllum* fruits in white colour (left) and ripen *C. odontophyllum* fruits turned purple in colour** (right)

[Adopted from Ref. 32]

C. odontophyllum fruits of superior quality are distinguished by their plumpness, weighing approximately 18 grams apiece, possessing thick and yellow flesh, emitting a pleasant nutty fragrance, and having a smooth and creamy texture that provides a neutral or slightly acidic flavor. These characteristics render them exceptionally desirable. Typically, *C. odontophyllum* fruit has a short shelf life of three days. Its shelf life at room temperature is limited to three days, as moisture loss causes the skin to wrinkle [33]. The alterations in appearance diminish the palatability, indicating additional decline. This attribute presents a substantial obstacle for the dissemination and promotion of *C. odontophyllum* fruit on both a domestic and global scale.

3.4. Phytochemical constituents

Scientific studies that have undergone peer review have provided several pieces of data indicating the advantageous effects of extracted chemicals derived from different portions of the *C. odontophyllum* plant [34]. The *C. odontophyllum* fruit contains a substantial amount of bioactive chemicals in all its sections, including the pulp, skin, and kernel. The *C. odontophyllum* fruit is composed of many components that contain phytochemicals such as flavonoids, tannins, and terpenoids. These compounds have been linked to a variety of positive effects on health. The benefits encompass the reduction of cholesterol levels, decreased risk of atherosclerosis, inhibition of cholinesterase activity, display of antibacterial characteristics, and probable provision of anti-diabetic effects [8]. The chemical structure of the phytoconstituents of *C. odontophyllum* fruit are given in Table S1 (Supplementary).

3.4.1. Phenolic compounds (flavonoids and anthocyanin)

Research has demonstrated the antioxidant properties of various parts of the *C. odontophyllum* fruit, including the skin, flesh, and kernel [35]. *C. odontophyllum*, especially the outer skin has been found to contain high levels of antioxidant compounds, such as phenolics, flavonoids, and anthocyanins, making it a significant source of antioxidants [36]. C. odontophyllum's skin emerges as a major source of antioxidants due to its high phenolic content, whereas the kernels have the lowest levels of phenolics [34]. Flavonoids, a type of compound found in *C. odontophyllum*, have been shown to offer various health benefits. They serve as natural antioxidants in diets, helping to neutralize the harmful effects of free radicals and thereby contribute to the prevention of certain disorders. Additionally, flavonoids interact with various cellular targets, exhibiting anti-inflammatory, antiviral, antibacterial, anti-aging, and even anticancer properties [37].

Research has also looked into the extraction of

antioxidant chemicals utilising ethylacetate, butanol, and water from the peel, pulp, and seeds of *Canarium odontophyllum*. According to Prasad et al. (2011a), the peel's ethyl acetate fraction had the highest concentration of phenolic and flavonoid compounds and shown robust antioxidant properties that were on par with those of commercial BHT antioxidants. These findings raise the possibility of using this extract as a natural antioxidant [38]. Furthermore, numerous studies confirm the existence of phenolic chemicals in *C. odontophyllum*. These substances, which include anthocyanins, flavonoids, and phenolic acids, have been found in the pulp, peel, and kernel of the fruit, among other sections [39]. The fruits included ethyl gallate, five flavonoids (catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, and apigenin), two phenolic acids (ellagic and vanillic acids), and ethyl gallate. Furthermore, three anthocyanidins (cyanidin, pelargonidin and delphinidin) and four anthocyanins (malvidin-3,5-di-O-glucoside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-glucoside) were detected (Table 1) [40].

Notably, different districts in Malaysia where *C. odontophyllum* fruits are grown exhibit variations in nutritional composition and antioxidant properties. Areas with lower pollutant exposure tend to positively influence the phytochemical properties of *C. odontophyllum* fruits. Kapit, in particular, stands out for producing elite fruits with high levels of total phenolics, flavonoids, and anthocyanins, along with significant antioxidant activities [13]. The deep purple hue of *C. odontophyllum* peel is due to the presence of anthocyanins (Figure 6), which are a prominent class of phenolic compounds found in the peel. *C. odontophyllum* peel has been shown to contain different varieties of anthocyanins, including cyanidin glucoside, malvidin glucoside, and peonidin glucoside [40]. These anthocyanins contribute to the fruit's antioxidant properties and its appealing color.

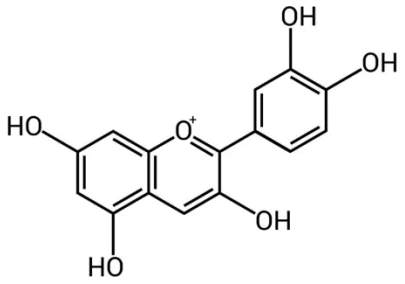

Figure 6: **Structure of anthocyanin**

3.4.2. Carotenoids

Carotenoids are lipophilic compounds that are responsible for red, orange and yellow hues of fruits and vegetables [41]. Numerous fruits and vegetables contain these chemicals, which have been demonstrated in studies to help lower the risk of heart disease, several malignancies, and other health issues [42]. Carotenoids from underutilized fruits have recently attracted more attention, especially from rural communities [43]. The two classes of carotenoids are xanthophylls and carotenes. The most prevalent form of carotene in nature is β-carotene, which gives objects a yellow-orange color. The main phytochemical that gives some fruits their orange-red color is lycopene. According to Khoo et al. (2011), lutein, a xanthophyll, is generally found in green leafy vegetables but is also occasionally found in fruits. The main carotenoid in plants is all-trans β-carotene, which is essential to the antioxidant defense mechanism of the plant [44]. All-trans β-carotene is the predominant carotenoid in plants, playing a crucial role in the plant's antioxidant defense system. Some green-colored fruits may contain a high amount of carotenoid because the yellow-orange-colored carotenoid pigments are masked by chlorophylls [45].

A study conducted by Prasad et al. (2011) examined *C. odontophyllum* Miq. and detected carotenoids from its peel, pulp, and seed fractions (Table 1) [38]. The peel had the greatest concentration of all-trans-β-carotene, with the pulp and seed following suit. Furthermore, 15-cis-β-carotene, 9-cis-β-carotene, and 13-cis-β-carotenes played

a significant role in the carotenoid levels found in the peel, pulp, and seed portions. The pulp shown remarkable β-carotene bleaching and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging properties, whilst the peel revealed superior scavenging action against 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radicals. All samples had a significant inhibitory impact on the oxidation of haemoglobin produced by hydrogen peroxide. The findings of this study indicate that the fruit of *C. odontophyllum*, due to its abundant carotenoid content and antioxidant characteristics, has the potential to serve as a natural antioxidant. It might be utilized in the development of functional foods to offer various health advantages.

The fruit of *C. odontophyllum* contains a significant amount of phenolics, flavonoids, anthocyanins, and carotenoids, making it a promising source of natural antioxidants and acetylcholinesterase inhibitory agents. Carotenoid-rich meals have been found to provide protection against cancer, heart disease, and diabetes, as indicated by epidemiological research [43]. Carotenoids, which are responsible for the pigmentation of fruits, result in a greater concentration of carotenoids in the outside flesh of the fruit as opposed to the seed. Previous studies on the carotenoid profiles of the peel, pulp, and seed of *C. odontophyllum*, as well as their antioxidant capacities, found that the peel and pulp contained a higher amount of all-trans-β-carotene (Figure 7) compared to the seed [46].

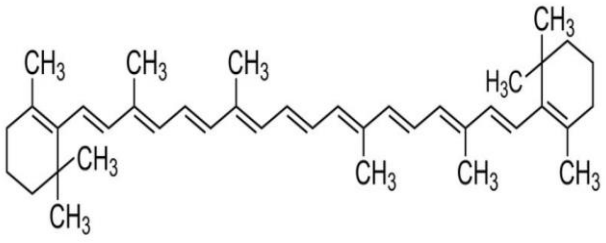

Figure 7: Structure of beta-carotene

3.4.3. Terpene and terpenoids

Terpenoids are a broad and varied group of naturally occurring organic compounds that are linked to terpenes [47]. Terpenes and terpenoids are organic compounds found naturally in plants. Fruit includes many types of terpenes, including monoterpene, triterpene, and sesquiterpene. In addition to carotenoids, saponin is a type of terpenoid that is present in the defatted *C. odontophyllum* fruit [48]. Terpenoids, particularly saponins, play a crucial role in combating infectious diseases, functioning as potent antimicrobial agents. According to Murugeasn et al. (2010), the majority of terpenoids, including saponins (Figure 8), have the potential to act as antioxidants (Table 1) [49]. In addition to their antioxidant activity, saponins offer various health benefits [50].

Studies on *C. odontophyllum* leaves have identified flavonoids, tannins, terpenoids, and phenols. The results indicate that the leaves of *C. odontophyllum* may possess therapeutic chemicals that could be effective against *S. aureus*, offering a viable alternative treatment for skin and soft tissue infections caused by *Staphylococcus aureus* [51].

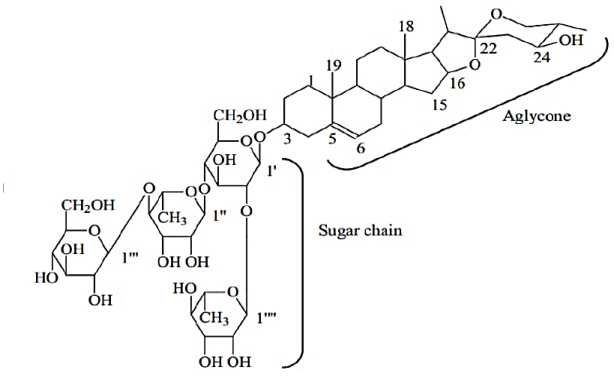

Figure 8: **Structure of saponin**

Table 1. Phyconstituents of *Canarium odontophyllum* fruit

Type [34]	Sub type	Compound
Flavonoids	Flavonoids	Catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, and apigenin [40]
	Anthocyanidins	Cyanidin, pelargonidin and delphinidin [43]
	Anthocyanins	Malvidin-3,5-di-O-glucoside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-glucoside [40]
	Phenolic acids	Ellagic and vanillic acids [40]
Carotenoids	All-trans-β-carotene	15-cis-β-carotene, 9-cis-β-carotene, and 13-cis-β-carotenes [40]
Terpenoids	Saponins [49]	--

3.5. Nutritional values

3.5.1. Proximate compositions

Proximate analysis serves as a method to quantify the components of food substances, encompassing parameters such as ash, moisture, crude protein, total fat, total carbohydrates, and dietary fiber [52]. The *C. odontophyllum* fruit is known for its high nutritional value, containing 339 kcal of energy per 100 g of edible portion. It also contains significant amounts of protein (3.8%), fat (26.2%), carbohydrates (22.1%), crude fibre (4.3%), ash (2.3%), phosphorous (65 mg per 100 g of edible portion), potassium (810 mg per 100 g of edible portion), calcium (200 mg per 100 g of edible portion), magnesium (106 mg per 100 g of edible portion), and iron (1.3 mg per 100 g of edible portion) (Table 2) [21]. The fruit's oil possesses significant antioxidant capabilities [27].

The nutritional composition and antioxidant properties of *C. odontophyllum* Miq. fruits were investigated in a study undertaken by Chew et al. throughout various districts in Malaysia, namely Kanowit, Sarikei, Kapit, and Song in Sarawak. *C. odontophyllum* fruits from different districts exhibited variations in their nutritional composition and antioxidant characteristics, mostly due to regional reasons. *C. odontophyllum* fruits exhibited enhanced phytochemical qualities in areas with less pollution exposure. The red *C. odontophyllum* fruit discovered in Sarikei has notable distinctions from the typical purplish-black *C. odontophyllum* fruit. The ash, moisture, and fat levels of *C. odontophyllum* fruit remain consistent throughout different growing zones. However, the carbohydrate and protein contents of *C. odontophyllum* fruit exhibit variations depending on the specific region in which they are grown [13]. In addition, the carbohydrate content of *C. odontophyllum* fruit gathered from Sarikei (9.16%) and Song (8.97%) was approximately twice as high as that of Kanowit (4.45%) and Kapit (5.07%). However, there was only a little variation in the protein content of *C. odontophyllum* fruit taken from different places in Sarawak. The primary macronutrient present in *C. odontophyllum* fruit is fat, and its composition remains consistent regardless of the variations in growing locales. The red *C. odontophyllum* fruit type obtained from Song, Kapit, and Kanowit is a rich source of carbohydrates, although it contains less protein compared to the purple variation [53]. Investigation of the edible portions (peel, pulp, and kernel) revealed that *C. odontophyllum* peel had higher ash and moisture content, while the kernel exhibited the highest percentage of fat and protein [54].

Table 2. Nutritional value of *Lepisanthes fruticose* fruit

Nutrition	Quantity
Protein	3.8%
Fat	26.2%
Carbohydrates	22.1%
Crude Fibre	4.3%
Ash	2.3%
Phosphorous	0.65 %
Potassium	0.81%
Calcium	0.20%
Magnesium	0.11%
Iron [21]	0.001%
Palmitic Acid	--
Oleic Acid	--
Linoleic acid [36]	--
Iron	0.002 – 0.003%
Zinc	0.00077-0.00092%
And Copper [13]	0.00021-0.00047%

C.odontophyllum kernel is known for its significant di etary fibre content, which can reach to 22%. This attribute is linked to several health advantages, such as a decreased likelihood of developing type 2 diabetes and heart disease [16]. Dietary fibre refers to the parts of plants that can be eaten but cannot be broken down or absorbed by the digestive system. Dietary fibre can be classified based on its ability to be digested in the small intestine into two types: soluble and insoluble dietary fibre. In a study, it was discovered that all genotypes of *C. odontophyllum* fruit exhibit minimal amounts of soluble dietary fibre (ranging from 0.00 to 0.16 g/100g) and significant amounts of insoluble dietary fibre (ranging from 9.29 to 22.98 g/100g) (Table 2) [13].

3.5.2. Fatty acid and amino acid compositions

The *C. odontophyllum* fruit is notable for its abundant and diverse nutritional content. A single *C. odontophyllum* fruit weighing 100 grams contains 339 kilocalories of energy, consisting of 26.2 grams of fat, 22.1 grams of carbohydrates, and 3.8 grams of protein [55]. This attribute renders it a fruit with a high caloric density, particularly due to its elevated fat content in comparison to other fruits with high fat content, such as olive (23 g/100g) and avocado (25 g/100g).

The nutritional value, physical qualities, and stability of fat are influenced by the compositions of fatty acids. The primary fatty acids found in *C. odontophyllum* pulp and kernel oils are palmitic acid (16:0), oleic acid (18:1), and linoleic acid (18:2). Azlan et al. discovered that palm oil and *C. odontophyllum* pulp oil had comparable fatty acid compositions, with around 40% saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), as well as approximately 12-13% polyunsaturated fatty acids (PUFAs) [36]. Despite the overall high fat content of *C. odontophyllum*, the MUFAs in *C. odontophyllum* pulp oil, at 42%, are notably lower than those in avocado oils (65-68%), as indicated by studies conducted by Shakirin et al. (2010) [54] and Azlan et al. (2010) [36].

Further studies by Kadir et al. (2021) revealing the presence of saturated fatty acid oleoresin in *C. odontophyllum* pulp [56]. The *C. odontophyllum* pulp has been scientifically demonstrated to have low levels of peroxide and free fatty acids, while being abundant in vitamin E (α-tocopherol) [55]. The study conducted by Kadir et al. (2021) investigated the potential of *C. odontophyllum* pulp oil (DPO) and defatted *C. odontophyllum* pulp (DDP) to reduce cholesterol levels and provide hepatoprotective effects in hypercholesterolemic rats [57]. DDP, which is rich in anthocyanin and syringic acid, has been shown to effectively reduce cholesterol, low-density lipoprotein, and β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase, an enzyme that is vital in the formation of cholesterol.

The acid value, peroxide value, iodine value, and saponification value of the extracted crude oils from both *C. odontophyllum* fruit flesh and kernel were analyzed [33]. The results showed that the characteristics of the crude oils obtained from both *C. odontophyllum* fruit flesh and kernel were similar to those of commercially available palm and olive oil. The *C. odontophyllum* meat oil exhibited a yellowish hue and displayed physicochemical

parameters that closely resembled those of olive oil. The elevated acid concentration in *C. odontophyllum* meat oil can be linked to the presence of unbound fatty acids in its crude state, which contrasts with the reduced acid content seen in commercially available palm and olive oils that have undergone refining processes. In addition, the protein found in *C. odontophyllum* fruit has been shown to be particularly abundant in aspartic and glutamic acids, which make up 45-49% of the total amino acids present [13].

3.5.3. Minerals and vitamins composition

Minerals and vitamins are essential micronutrients necessary for the proper functioning and overall health of the organism. *C. odontophyllum* is a highly nutritious fruit, abundant in energy, protein, and potassium [55]. In addition, the research conducted by Chew, L. Y. et al. also indicated that lipids are the main macronutrient in *C. odontophyllum* fruits, with calcium, sodium, and potassium being the prominent minerals [13]. The *C. odontophyllum* fruit contains a significant amount of calcium (16.00–67.88 mg/100 g fresh weight (FW)), salt (7.26–11.19 mg/100 g FW), and potassium (3.64–7.19 mg/100 g FW) [16]. However, the mineral concentrations showed substantial variation based on geographical location and variety [13]. However, Hoe and Kueh's study revealed that *C. odontophyllum* fruit contains high levels of potassium (810 mg per 100 g of edible portion), calcium (200 mg per 100 g of edible portion), magnesium (106 mg per 100 g of edible portion), and phosphorus (65 mg per 100 g of edible portion), making them the most abundant minerals in the fruit [55].

However, conflicting findings exist regarding the impact of geographic regions on fruit mineral compositions. While some studies [58] assert a strong influence of geographic regions on the mineral compositions of fruit, others, like [59], report no such effect. The locations and cultivation variations of *C. odontophyllum* fruit have a significant impact on its mineral content. Specifically, iron (2.10-3.14 mg/100g), zinc (0.77-0.92 mg/100g), and copper (0.21-0.47 mg/100g) are regarded as minor minerals, while calcium (28.47-43.72 mg/100g), sodium (5.02-6.93 mg/100g), and potassium (5.02-6.93 mg/100g) emerge as important minerals (Table 2) [13]. All things considered, these differences highlight how crucial it is to take location and diversity into account when evaluating the mineral content of *C. odontophyllum* fruit. Overall, these variations underscore the importance of considering both variety and location when assessing the mineral composition of *C. odontophyllum* fruit.

3.6. Pharmacological properties

3.6.1. Antioxidant

Studies were conducted on the antioxidant potential of the skin, pulp, and seed of the *C. odontophyllum* fruit (Table 3) [54]. The carotenoid makeup of these fruit components was investigated in a separate 2011 study by Prasad et al. [38]. Furthermore, Chew and colleagues investigated the possible antioxidant characteristics of *C. odontophyllum* fruits, both purple and red [13]. According to a study conducted by Chew et al., *C. odontophyllum* fruit has an impressive antioxidant capacity of 0.68 ± 0.09 mmol Trolox equivalents per gramme of dry weight. The fruit's potential to considerably postpone the oxidation process of compounds susceptible to oxidation is indicated by the high concentration of antioxidants found in *C. odontophyllum*. This discovery is especially noteworthy since it suggests possible therapeutic advantages in treating a number of illnesses, such as cancer, liver problems, and cardiovascular ailments [13].

3.6.2. Anti-cholinesterase

Acetylcholinesterase, an important enzyme that breaks down acetylcholine, is found in *C. odontophyllum* fruits. In order to protect the brain system against the depletion of cholinergic neurons—a situation linked to diseases like Alzheimer's disease—acetylcholinesterase is essential. Cognitive performance declines as cholinergic neurons gradually disappear in Alzheimer's disease. Acetylcholinesterase has been found in *C. odontophyllum* fruits, which may indicate that the enzyme plays a

supportive role in preserving the integrity of the nervous system and may have implications for lessening the effects of neurodegenerative illnesses, especially those that include failure of the cholinergic system.

3.6.3. Cholesterol-lowering

Ensuring a nutritious diet is essential for effectively controlling cholesterol levels in the human body. The *C. odontophyllum* fruit has exhibited properties that can combat obesity and lower cholesterol levels, although the specific effects may differ depending on the type of cultivar. Azlan et al. (2013) have shown that *C. odontophyllum* pulp oil plays a crucial role in lowering triglyceride and total cholesterol levels (Table 3) [60].

Extracts from *C. odontophyllum* have demonstrated effectiveness in reducing plasma cholesterol levels, thereby lowering the level of low-density lipoprotein (LDL), a significant contributor to atherosclerosis development in arteries. In a study conducted over a period of eight weeks, rabbits that were fed a diet high in cholesterol were given a defatted *C. odontophyllum* extract at a concentration of 5%. The results showed that this extract led to a decrease in both total cholesterol and LDL-cholesterol levels when compared to a control group [61]. Additionally, rabbits fed with a high-cholesterol diet and defatted *C. odontophyllum* pulp experienced a significant increase in high-density lipoprotein levels [62]. In addition, the atherosclerotic plaques in the rabbit group that was fed a high-cholesterol diet and supplemented with defatted *C. odontophyllum* extracts showed a decreased severity compared to the control group. Hence, the extracted defatted *C. odontophyllum* fruit is considered a promising nutraceutical resource owing to its antiatherosclerotic characteristics. Although animal studies have shown encouraging outcomes, there is currently no human-based research available to confirm the cholesterol-lowering properties of defatted *C. odontophyllum* extract. To fill this need, it is advisable to conduct a human intervention study in future research, particularly due to *C. odontophyllum*'s potential for commercialization as an underutilized fruit that offers substantial health advantages.

Beta-carotene has been studied as a potential preventive agent in the atherosclerosis process due to its recognition of the significance of low-density lipoprotein (LDL) oxidation [46]. Epidemiological studies have indicated that there is a negative relationship between levels of beta-carotene in the blood and fat tissue, and the likelihood of developing coronary heart disease. However, randomized clinical trials have not consistently demonstrated the advantages of taking beta-carotene supplements, and in certain instances, have even suggested potential harmful consequences. The inconsistency between trials and epidemiological evidence may be influenced by various factors, such as correlations with other carotenoids in diet and blood, as well as the presence of other plant-derived compounds. Although the trial results do not substantially support the idea that beta-carotene can prevent heart disease, the overall epidemiological evidence indicates that consuming a diet abundant in foods high in carotenoids is linked to a decreased risk of heart disease. In view of this, *C. odontophyllum* fruits which are rich in carotenoid contribute to cholesterol lowering, reducing the risk associated with heart disease.

3.6.4. Antibacterial

An acetone shell extract derived from the seeds of *C. odontopyhllum* fruit demonstrated antimicrobial activity, particularly with bactericidal effects against Acinetobacter baumannii. While its effectiveness against inhibiting the growth of *P. mirabilis* was relatively lower, the antimicrobial properties of the acetone shell extract were attributed to the presence of terpenoids and tannins among its phytochemical components. These findings suggest that the extract has promising potential as an alternative phytotherapeutic approach for addressing a range of bacterial infections (Table 3) [63]. In a different investigation, Basri et al. investigated the antibacterial properties of distilled water, acetone, and methanol

extracts from the *C. odontophyllum* tree's stem bark against a variety of microbes. According to the study, *Acinetobacter baumannii* was bacteriostatically affected by the methanol and acetone extracts of the *C. odontophyllum* stem bark, while Staphylococcus aureus was bactericidally affected by them (Table 3) [64].

Table 3. Phamacological activity of *Lepisanthes fruticose* fruit

Parts	Compounds	Pharmacological/Biological Activity
Pulp, seed	Carotenoids	Antioxidant [54]
Pulp oil	--	Lowering triglyceride and total cholesterol levels [60].
Acetone extract of fruit	Terpenoids and tannins	Antibacterial [63]
Water and methanol extract of fruit	--	Antibacterial [64]
Hexane and acetone extract of pulp	Flavonoids	Antifungal [65]

3.6.5. Antimalarial

It is imperative that chemicals produced from plants be explored in order to develop antimalarial medications. Notably, vital antimalarial drugs like quinine and artemisinin have been derived from plant sources, underscoring the critical role that botanical contributions have played in the advancement of this discipline. Ishak et al. (2020) have researched *C. odontophyllum* leaf extracts' potential as an antimalarial drug in great detail [66]. The study showed that the methanol extract of *C. odontophyllum* leaf might hinder the growth of plasmodium when it reaches 5% parasitemia, affecting several phases of its development, such as young trophozoite, mature trophozoite, and schizont. This discovery implies the potential for advancing *C. odontophyllum* leaf extract as a pharmaceutical treatment for malaria. Shamsuddin et al. (2021) made a noteworthy contribution to this field by demonstrating that the combination of *C. odontophyllum* leaf extract and acetone provides valuable information about the antimicrobial mechanism. This research has the potential to identify a specific protein target for the development of new therapies against methicillin-resistant Staphylococcus aureus infections [67].

3.6.6. Antifungal

The study conducted by Chew et al. (2011) confirmed the existence of flavonoids (catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, and apigenin) and anthocyanidins (cyanidin, pelargonidin, and delphinidin) in the fruit of *C. odontophyllum* [13]. Flavonoids, which are frequently present in different plant components such as fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis, and honey, have been used for ages in the treatment of human ailments because of their physiological effects. Due to the extensive capacity of flavonoids to hinder the development of spores from plant diseases, flavonoids have been suggested for their potential application in combating fungal pathogens in humans. The existence of flavonoid in *C. odontophyllum* suggests that flavonoid could be the active component accountable for the antifungal activity. Extensive research has been conducted on the antibacterial properties of *C. odontophyllum* fruit. Basri et al. (2014) found that extracts of hexane and acetone from the pulp of *C. odontophyllum* have antifungal properties against *Candida glabrata*. This suggests that *C. odontophyllum* fruit has the potential to be used as an anti-yeast agent (Table 3) [65].

3.6.7. Anticancer

C. odontophyllum is a natural plant that shows promise in its potential to have anticancer effects. The acetone extract obtained from the stem bark of *C. odontophyllum* exhibits greater sensitivity towards HCT 116 in comparison to HT 29. The extract's ability to inhibit the

growth of HCT 116 and HT 29 cells suggests that it could be used as an anticancer drug for treating colorectal cancer [68]. The acetone extract derived from the stem bark of *C. odontophyllum* has great potential in the development of an anticancer drug that specifically targets HCT 116 cells. Importantly, this extract does not have any harmful effects on human colon fibroblast cells [69].

Attempts have been undertaken to establish a connection between the antioxidant characteristics of phytoextracts and their potential for combating cancer. Although there is no definitive proof of such a connection, it is proposed that the antioxidant properties of phytoextracts could be an indication of their ability to prevent cancer. The extract of the leaves of *C. odontophyllum* contains terpenoid, tannin and flavonoid. The absence of alkaloids in the leaves of C. odontophyllum suggests that chewing the leaves or consuming *C. odontophyllum* tea may be considered safe for use as a chemo preventive supplement against colon cancer. The cytotoxic activity of the leaf extracts may be correlated with the phytoconstituents, as flavonoids, tannins, and terpenoids are recognized for their protective role as antioxidants [70]. The importance of tannins and flavonoids as anticancer agents is supported by a study where the ethanol extracts of leaves from *Canarium patentinervium* Miq. exhibited radical scavenging activities primarily attributed to the presence of tannins and flavonoids [71].

3.6.8. Anti-hyperglycemic

The aqueous extract derived from the leaves of *C. odontophyllum* Miq. has exhibited the capacity to decrease blood glucose levels in mice with diabetes [72]. In a 4-week research, it was found that the *C. odontophyllum* extract, when administered at a concentration of 600 mg/kg bw, had a notable effect in reducing plasma glucose levels compared to a dose of 300 mg extract/kg bw. Nevertheless, the *C. odontophyllum* extract did not elevate the insulin level, but it did enhance insulin sensitivity and alleviate insulin resistance (HOMA-IR). The finding suggests that the *C. odontophyllum* extract has been found to have antihyperglycemic characteristics, which means it can temporarily lower glucose levels [73].

4. CONCLUSION

The various components of *C. odontophyllum*, such as its skin, flesh, and seed, possess distinct nutritional attributes, each exerting specific health benefits. With its increasing significance, *C. odontophyllum* is poised to become a key crop in Sarawak, gaining recognition alongside other globally esteemed fruits. This suggests a potential improvement in its position within the global market. The economic implications are considerable, as *C. odontophyllum* is expected to play a major role in driving the nation's GDP growth. In essence, *C. odontophyllum* emerges not only as a promising agricultural commodity but also as a multifaceted resource with implications for health, biodiversity, and economic development. The unique combination of nutritional richness, health benefits, and economic viability underscores its potential to become a valuable asset both locally and globally.

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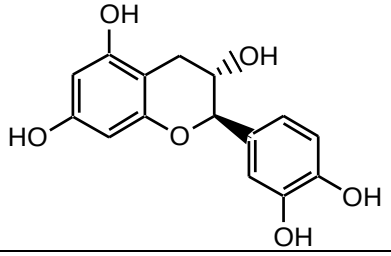
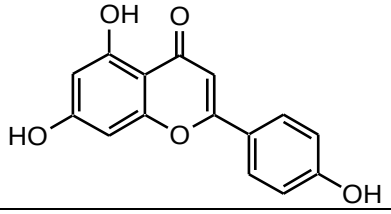
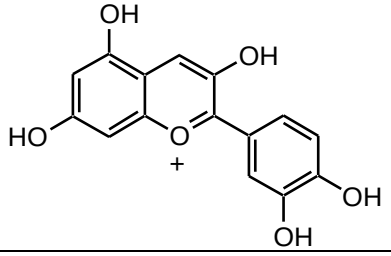
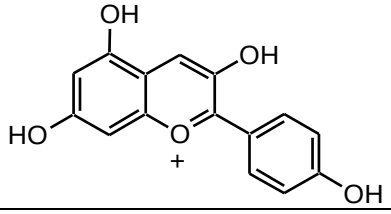
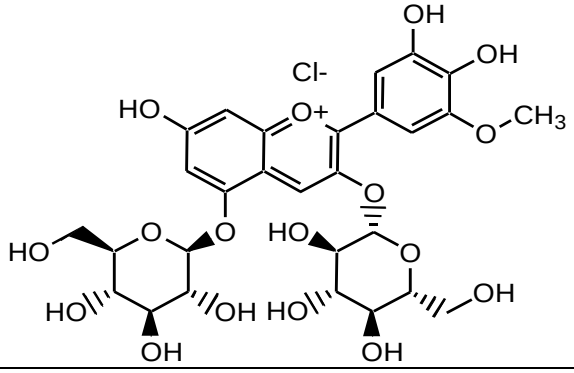
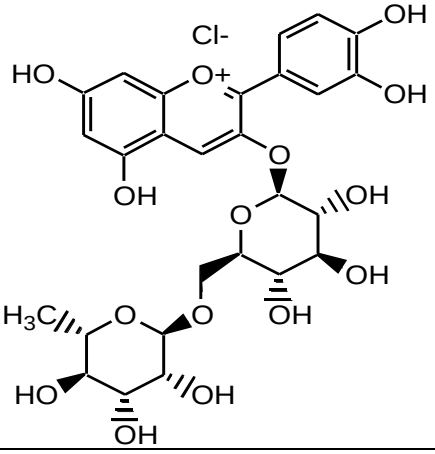
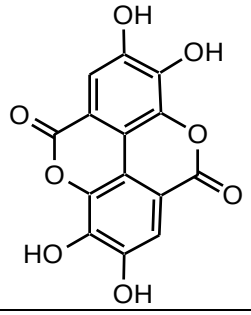
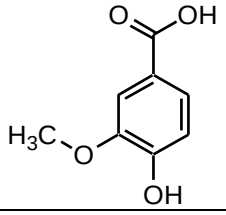
Conflict of interest statement

The authors declare that there is no conflict of interest.

Table S1: Chemical structures of phytoconstituents of *Canarium odontophyllum* fruit

Compound	Chemical Structures
Catechin	
Apigenin	
Cyanidin	
Pelargonidin	
Malvidin-3,5-di-O-glucoside	

Compound	Chemical Structures
Cyanidin-3-O-rutinoside	
Ellagic acid	
Vanillic acid	

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مراجعة شاملة لفواكه *Canarium odontophyllum* وفوائدها المتعددة الأوجه

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ملخص

تم العثور على نبات Canarium odontophyllum Miq النادر، والمشار إليه باسم "dabai" باللغة المحلية، في ساراواك، ماليزيا. غالبًا ما يتم استهلاك الفاكهة بسبب فوائدها الصحية المزعومة، ولكن لا يتم استغلالها بشكل كافٍ بسبب مدة صلاحيتها القصيرة، خاصة في المناطق الريفية. على الرغم من كونها فاكهة موسمية، إلا أن داباي معروفة بقيمتها الغذائية، وقد بحثت بعض الدراسات الطبية في السمات البيولوجية لنبات Canarium odontophyllum لدعم استخدام النبات كمكمل غذائي وصحي. إن لب فاكهة داباي وقشرها ونواتها كلها مواد مغذية للغاية ونشطة بيولوجيًا وتحتوي أيضًا على ألياف غذائية. تحتوي فاكهة داباي على مركبات الفلافونويد والعفص والتيربينويدات. بالإضافة إلى ذلك، أظهرت المستخلصات الخام من Canarium odontophyllum مجموعة متنوعة من الفوائد العلاجية، بما في ذلك التأثيرات المضادة للميكروبات ومضادات الملاريا ومضادات الأكسدة ومضادات السرطان ومضادات السكر والفطريات. يتميز داباي بمحتواه الغذائي العالي، بما في ذلك كميات كبيرة من البروتين والدهون والكربوهيدرات. تم البحث في قواعد البيانات الإلكترونية، مثل PubMed، وScopus، وWeb of Science، وGoogle Scholar، منذ بداية عام 1980 وحتى نهاية نوفمبر 2023 من أجل استخراج المنشورات ذات الصلة.

الكلمات الدالة: Canarium odontophyllum، dabai، الخصائص الفيزيائية، المكونات النباتية، القيم الغذائية، النشاط الدوائي.

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Antioxidant and Antimicrobial Potentials of *Nicotiana glauca* Graham Leaves Extracts and Synthesized Silver Nanoparticles: A Phytochemical Approach

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ABSTRACT

Nicotiana glauca is a medicinal plant that belongs to the genus *Nicotiana*, traditionally used for the treatment of many diseases. This study aims to screen the phytochemical content of leaf extracts of *Nicotiana glauca*, synthesize silver nanoparticles using the extracts, and evaluate their antioxidant and antimicrobial activities. The leaf samples were collected, air-dried, and ground into powder. The leaf powder was macerated with distilled water, methanol, n-hexane, and chloroform to extract the phytochemicals. Phytochemical analysis was performed using standard methods. Synthesis of silver nanoparticles was achieved by mixing a 3 mM silver nitrate solution with the plant extract, and the synthesized silver nanoparticles were characterized by X-ray diffraction and scanning electron microscopy. The antioxidant activities of the extracts and the synthesized silver nanoparticles were evaluated by the DPPH scavenging assay, while the in vitro antimicrobial activities were evaluated using the agar disc diffusion method against selected bacterial and fungal strains. The results of the phytochemical analysis indicated the presence of alkaloids, saponins, flavonoids, terpenoids, tannins, phenolics, steroids, and glycosides. The results of the antioxidant activity evaluation of AgNPs, methanol extract, chloroform extract, and n-hexane extract showed that they possess remarkable antioxidant activities. The antioxidant activity analysis also indicated that percentage inhibition and IC50 were dose-dependent. Synthesized silver nanoparticles showed the highest antioxidant activity with an IC50 value of 78 µg/mL, while the methanol extract gave an IC50 value of 170 µg/mL. The results of the antimicrobial activity evaluation showed that the plant extract and the synthesized silver nanoparticles exhibited antimicrobial activities. The highest zone of inhibition observed was 16.33±1.155 mm for synthesized silver nanoparticles and 15.33±1.155 mm for the plant extract. The lowest zone of inhibition observed was 9.67±0.577 mm for synthesized silver nanoparticles and 7.33±0.577 mm for the plant extract. Generally, the plant extracts and synthesized silver nanoparticles exhibited strong antioxidant and antimicrobial activities. Further studies should be conducted on the phytochemical constituents, antioxidant, and antimicrobial activities of this plant.

Keywords: Antimicrobial; Antioxidant; *Nicotiana glauca*; Phytochemical; Silver nanoparticle.

1. INTRODUCTION

Medicinal plants have been used as a source of medicine for human and animal diseases for a long time [1]. Ethiopia is one of Africa's 6th plant-rich countries, with around 60% of the plants being indigenous and most of them having medicinal properties [2]. In Ethiopia, only 10% of medicinal plant species are cultivated today, but a more significant number are left under wild stands threat [3]. Now a day medicinal plant treatments are still used for many health problems because they are safe, less toxic, economical, and a reliable key natural resource of drugs all over the world [4].

Plants contain different chemical components, such as phytochemicals, essential oils, seed oils, and others, which are important for various scientific applications and

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pharmaceutical uses [5]. Phytochemicals are bioactive chemicals that are produced by plants. They are considered secondary metabolites because plants need them in small amounts. In addition, they are naturally synthesized in all parts of the plant body: bark, leaves, stem, root, flower, fruits, and seeds [6]. Plants are rich in many secondary metabolites that have been found to demonstrate antimicrobial, antioxidant, and anti-infectious properties [7]. Phytochemical screening of medicinal plants is important in discovering new sources of therapeutically and industrially important compounds [8].

One of the current problems in treating infectious diseases is the development of antimicrobial resistance. Microbial resistance against antibiotics is the ability of microorganisms to tolerate the effects of drugs that inhibit their growth. Microorganisms changed as a result of responding to drugs is the main reason for the resistance [10]. Searching for other ways to overcome the antimicrobial resistance of secondary metabolites is one alternative.

The Indigenous people have a long history of using plants for medicinal purposes. Despite the increasing acceptance of traditional medicine in Ethiopia, this rich indigenous knowledge is not sufficiently documented. Documentation of plants used as traditional medicines is needed so that the knowledge can be preserved, and the utilized plants can be conserved and used sustainably. In addition, the studies on the therapeutic value of plants are not that well done [9].

An antioxidant is the activity of many vitamins, minerals, and other phytochemicals. Antioxidants are used to protect the body against the damage caused by reactive oxygen species (ROS). Reactive oxygen species (ROS) can react with the body and damage many structures in the body [11]. Reactive oxygen species (ROS) cause many psychological disorders; some common examples are atherosclerosis, heart disease, aging, diabetes mellitus, immunosuppression, nervous disorders, and others. Many types of synthetic antioxidants are produced, and various food supplements containing antioxidants are there to regulate the disorder that comes from reactive oxygen species (ROS), but these synthetic antioxidant capsules and dietary supplements are found to be less effective in many cases. To fulfill the thrust of antioxidants, many more medicinal plants contain natural antioxidants that have beneficial therapeutic potential. Some studies have shown that plants produce potent phytochemicals that serve as antioxidants. The majority of the antioxidant activity is due to the presence of phytochemicals such as flavones, isoflavones, flavonoids, anthocyanin, catechins, isocatechins, phenolic compounds, and tannins [12].

Many studies have shown that various plants are rich sources of antioxidants. Antioxidants include vitamins A, C, and E, as well as phenolic compounds found in plants such as flavonoids, tannins, and lignins. The consumption of fruits and vegetables has been linked with several health benefits as a result of their medicinal properties and high nutritional value [13].

Antioxidants have the ability to control and reduce oxidative damage caused by reactive oxygen species (ROS) in foods, by inhibiting their oxidation which ultimately increasing the shelf-life and quality of these foods. Antioxidants such as Beta-carotene, ascorbic acid, and many phenolics play roles in human health such as reducing inflammation, preventing certain cancers and even delaying aging. Due to this, increasing the consumption of fruits and vegetables which are rich in antioxidants has been recommended by many researchers throughout the world [14].

Another alternative solution for the treatment of infectious diseases is the use of nanoparticles. Silver is known as one of the elements nowadays used in modern nanotechnology. The green synthesis of silver nanoparticles (AgNPs) got the attention of many researchers because of their noted properties, such as good conductivity, catalytic activity, and antimicrobial effect [15]. The excellent activities of silver nanoparticles

depend on their size, shape, and morphology. Metals and nanoparticles have shown good antimicrobial activity. but AgNPs have shown better antibacterial, antifungal, and antiviral activity than other nanoparticles [16].

Nicotiana glauca is one of the medicinal plants that belongs to the family Solanaceae and used in Ethiopian traditional medicine. This plant comes from South America and is distributed throughout the world. It is invasive and mainly found on the roadside. It is a flowering plant has toxic property humans and animals. *Nicotiana glauca* has the local name as "yeareb kitel" in Amharic and is widely used by traditional healers as an antibacterial, antifungal, and antiviral. It is used to treat burns and inflammatory diseases and shows good biological activities, such as allelopathic activity [17, 18]. Although it is traditionally used as medicine, its phytochemical contents and antimicrobial and antioxidant activities are not well studied. Therefore, screening for phytochemical content and testing for the antioxidant and antimicrobial activity of this plant is very important to expose its potential medicinal properties. In addition studying the potential of this plant extracts to synthesize metallic nanoparticles is imperative. The aim of this study is phytochemical analysis, and antioxidant activities and antimicrobial activities evaluation of *Nicotiana glauca* leaf extracts, and leaf extract-mediated synthesized silver nanoparticles were evaluated.

2. MATERIALS AND METHODS

Description of the Study Area

The plant leaf samples were collected from Eteya town, Arsi zone, Oromia Regional State, Ethiopia. Eteya is the administrative center of Hetosa Wereda located about 170 km southeast from the capital, Addis Ababa. The geographical coordinates of Eteya town are 08^0 08ˈN, 39^0 14' E/ 8.133°N 39.233°E with an elevation range from 1500 to 4170 meters above sea level. The area covers 937 square km. It has 31% humidity, and its annual temperature ranges from 10-22 ^{0}C.1/ 8.133°N 39.233°E The area' has bimodal rainfall occurring from March to April is a short rain season and from July to October is a long rain season [19].

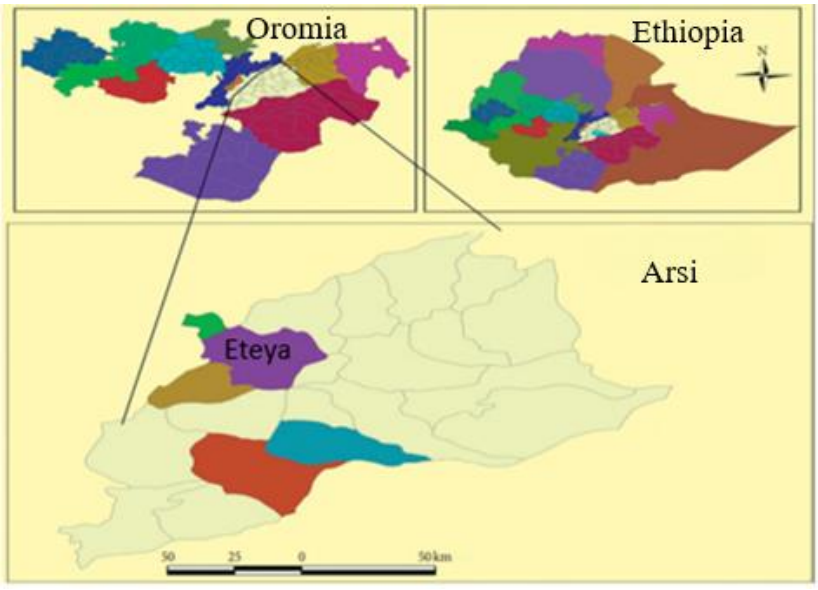


Figure 1. Maps of the study area [20]

Plant materials collection and identification

The plant materials of *Nicotiana glauca* were collected in January 2023 from fields and identified by botanist in the National Herbarium, Addis Ababa University. Voucher number (TG001) was given to the plant sample and the sample was stored in the National Herbarium, Addis Ababa University.

Preparations of the extracts

The collected leaves were washed with tap water to remove dirt and impurities and dried at room temperature in the shade for three weeks, The dried leaves were ground into powder using an electric grinder, and the leaf powder was weighted using an electronic balance and was kept in a flask with paper labeling. The leaf powder was weighted to 100 g using an electronic balance, and it was soaked (macerated) in 1000ml distilled water, methanol, n-hexane, and chloroform separately at room temperature for 72 hours with an electrical shaker at 120rpm. The crude extracts were filtered separately using Whatman No.1 filter paper. Then the filtrates were evaporated using a rotary evaporator, and the dried extracts were stored until used.

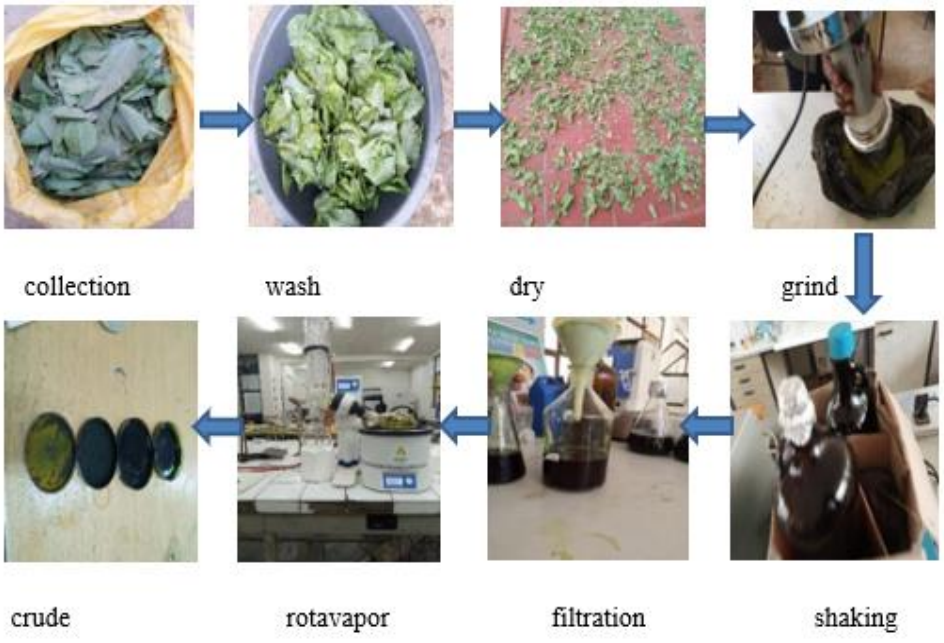


Figure 2. The extraction processes

Preparation of Silver Nitrate Solution

Silver nitrate solution was prepared by dissolving appropriate amount of silver nitrate (0.11466g) in 225 ml of double distilled water. The final concentration of the solution was made at 3mM.

Synthesis of silver nanoparticles using leaf extracts

The silver nitrate solution (3mM) and distilled water leaf extract were mixed in a 3:1 ratio, (225 ml of silver nitrate was mixed with 75 ml of plant extract). The mixture was heated to 60 °C for 1 hour using a magnetic stirrer. Before heating, the mixture was greenish black, any color change was recorded. The color of the mixture was changed gradually to brown which indicated the synthesis of silver nanoparticles (conversion of Ag^{+} to $Ag^{0)}$. Then, the mixture was centrifuged for 10 minutes at 13000 rpm. The synthesized silver nanoparticles settled at the bottom were washed three times with double distilled water and ethanol, dried in the oven for 24 hours at 50 °C and stored

for characterization and further activity [21].

Characterization of synthesized silver nanoparticles

The synthesized silver nanoparticles were characterized (size and morphology were determined) by SEM (Scanning Electron Microscope) and X-RD (X-ray diffraction) (SHIMADZU Corporation Japan) 7000 X-ray diffractometer. UV

Figure 3. Silver nanoparticle synthesizing process

Phytochemical screening

The qualitative Phytochemical analysis of the extracts was done with the following standard methods to determine the presence or absence of alkaloids, saponins, flavonoids, terpenoids, tannins, phenolics, steroids, and glycosides [22-28].

Test for alkaloids

The presence of alkaloids in the leaf extracts was tested by Wagner's test. 2 ml of the extract was added to a test tube, and it was treated with a few drops of Wagner's test reagent. The formation of a reddish-brown precipitate or turbidity shows the presence of alkaloids.

Test for saponins

Foam test: 5 ml of the extract was mixed with 20 ml of distilled water, and it was shaken in a graduated cylinder for 15 minutes. The formation of about 1 cm layer of foam indicates the presence of saponins.

Test for terpenoids: 5 ml of each extract was added to the test tube, and 2 ml of chloroform and 3 ml of concentrated sulphuric acid were added. The color change to red brown indicated the presence of terpenoids.

Test for flavonoids: 2 ml of extract was treated with a few drops of sodium hydroxide solution. Then it was observed in the form of an intense yellow color.

Test for tannins

Lead acetate test: 2 ml of extract was added to the test tube, and a few drops of 1% lead acetate were added. The formation of a yellowish precipitate was indicated by the

presence of tannins.

Test for phenolics

Ferric chloride test: 1 ml of the extract was added to the test tube, and a few drops of freshly prepared ferric chloride were added. The formation of a bluish-black color implies the presence of phenolic compounds.

Test for steroids

Liebermann test: 1 ml of the extract was dissolved in 2 ml of chloroform into the test tube, and 2 ml of acetic anhydride was added to the test tube. Then two drops of sulphuric acid were added. Finally, the color was changed into red, then blue, and finally green.

Test for glycosides

Small amounts of extract dissolved in 1 ml of distilled water, then 1 ml of sodium hydroxide was added to the test tube, and a yellow color was formed. This color change indicated the presence of glycosides.

Antioxidant activity test

The *in vitro* antioxidant activity evaluation of the leaf extracts and synthesized silver nanoparticles was done by testing their DPPH radical-scavenging activity using spectrophotometer (UV-VIS Shimadzu) as described before [29] with slight modification. That is, 1 ml of 1mM stock solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was prepared in methanol. The test solutions were prepared in different concentrations (50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL, and 250 µg/mL by dissolving in dimethyl sulfoxide and put in different test tubes at an equal volume. To these solutions, 2ml of DPPH solution was added, and the mixture was allowed to react at room temperature in the dark for 30 minutes. A blank sample was prepared by adding 100 µL of dimethylsulfoxide in the DPPH solution and used as negative control. Vitamin C (ascorbic acid) was used as a standard control. The experiments were done in triplicate. After 30 minutes, the absorbance of the solutions was measured at 517 nm, and the percentage scavenging activity was calculated using the following equation:

$$\text{Percentage Scavenging} = = \frac{A0-A1}{A0} x100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample extract. The IC50 values were calculated by linear regression plots. The IC50 value (concentration of sample where the absorbance of DPPH decreases 50% concerning absorbance of the control) of extracts was determined which correspond to the amount of each sample required to scavenge 50% of the DPPH free radicals.

Evaluation of antimicrobial activities

The antimicrobial activity of crude extracts and silver nanoparticles was evaluated on five microorganisms such as: Gram positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes), Gram negative bacteria (Pseudomonas aeruginosa, Escherichia coli)* and Fungai (*Candida albicans).* The identified test microorganisms were obtained from the Oromia regional laboratory, Adama. Ethiopia.

Bacterial inoculum preparation

Mueller-Hinton sterile agar plates were prepared, and the strains of bacteria: *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa,* and *Escherichia coli* were seeded on the plates. It was allowed to stay at 37°C for 24 hours. The inoculum suspension was prepared by picking several colonies of fresh Muller Hinton agar with a sterile inoculating loop and transferring the inoculum suspension into a test tube containing 5 mL of sterile Muller Hinton broth. The bacterial suspensions were adjusted to obtain turbidity visually comparable to the prepared 0.5 McFarland standards. The turbidity of the inoculum tube was adjusted to 0.5 McFarland standards ($1x10^8$cfu/mL) [30].

Fungal inoculum preparation

Sabouraund Dextrose (SDA) sterile agar plates were prepared, and the strain of fungus: *Candida albicans* was inoculated on the prepared plate. It was allowed to stay at 37°C for 48 hours. The inoculum suspension was prepared with a sterile inoculating loop and transferred inoculum suspended into a test tube containing 5 mL of Sabouraund Dextrose broth (SDB). The fungal suspension was

adjusted to obtain turbidity visually comparable to the prepared 0.5 McFarland standards. The turbidity of the inoculum tube was adjusted to 0.5 McFarland standards ($1x10^6$ cell/spores) [30].

Antimicrobial test

The standard disc diffusion method was used to evaluate the antimicrobial activities of leaf extracts and synthesized sliver nanoparticles. This method was used to test for the antimicrobial activities of the extract by measuring the zone of inhibition against the test organisms. Pure colonies of each test microorganism were diluted (0.1 ml) in sterile saline and the diluted test organisms were spread (swabbed) on Muller-Hinton agar (MHA) plates for bacteria and Sabouraund Dextrose (SDA) for the fungus by cotton swabs and allowed to dry. The extract was diluted by DMSO to make 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml concentrations. The silver nanoparticles were prepared in different concentrations: 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml. Sterilized filter paper disks (6mm in diameter) were impregnated with appropriate concentration of each plant extract and silver nanoparticles. The disks (made from Whatman No.1) were allowed to absorb the plant extracts and silver nanoparticles. The disks containing the plant extract and nanoparticles were transferred onto the surface of the seeded agar plates by using sterile forceps. The plates were kept at room temperature for 2 h for diffusion, and then it was incubated for 24 h at 37°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Dimethylsulphoxide (DMSO) was used as a negative control. Ciprofloxacin and Ketoconazole were used as positive controls for bacteria and fungi, respectively. The growth was compared with the reference as well as the control [31].

Determination of minimum inhibition concentration (MIC)

To determine the minimum inhibitory concentration of the extracts and silver nanoparticles, the previously described method was used [32]. Plant extracts serial dilutions were prepared from 200 mg/ml of the plant extract and 10mg/ml of silver nanoparticles using DMSO. The serial dilutions prepared for extracts were 100 ml, 50 ml, 25 ml, 12.5 ml, and 6.25 ml, and the serial dilutions prepared for silver nanoparticles were 10 mg/ml, 5 mg/m 2.5 mg/ml, 1.25 mg/ml, and 0.625 mg/ml. Five milliliters of sterile Muller-Hinton Broth and Sabouraund Dextrose Broth were prepared for bacteria and fungus, respectively. The test tubes were inoculated with 10µl of the adjusted inoculum bacteria and fungi suspension with their final inoculum sizes of 5×10^5 CFU/ml and 2.5×10^4 CFU/ml respectively. The test tubes were incubated at 37°C ± 1°C for 24 hr and observed for any visible microbial growth. The minimum concentration of the extract with no visible growth was considered as MIC value.

Determination of MBC and MFC

The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined after the determination of the MIC of bacterial and fungi. This was done by sub-culturing a sample from a test tube, yielding a negative microbial growth on Muller-Hinton Broth and Sabouraund Dextrose Broth for bacteria and fungi respectively. Then the broths were incubated according to growth requirement of each organism, to examine the presence or absence of microbial growth (turbidity). Finally, the lowest concentration of the extracts and silver nanoparticles that showed no turbidity (growth of microorganism) after incubation was considered as the MBC and MFC [32].

Data analysis

Antimicrobial activities were analyzed using SPSS software (SPSS 22 version). Descriptive statistics were used to summarize quantitative data. Comparisons between multiple numeric data were performed using one-way ANOVA and it was presented in mean±SD using tables. P values less than 0.05 were considered statistically significant. Microsoft Excel 2010 was used to analyze the antioxidant activity, and the results were presented by using tables and graphs.

3. RESULTS

The yield of leaf extracts of Nicotiana glauca

The extraction was done with four different solvents, such as methanol, distilled water, chloroform, and n-hexane. The results are shown in Table 2 below. 100g of plant powder was used in all solvents and the yield obtained from different solvent extractions were different (Table 1). The highest yield was obtained from Methanol extract, and the lowest yield was from n-hexane solvent.

Table 1. The percentage yield of extracts in gram

Solvent	The initial mass of the plant	Yield of extract	Percentage (%) yield
Methanol	100g	12.8g	12.8%
Distilled water	100g	7.3g	7.3%
Chloroform	100g	6.5g	6.5%
n-hexane	100g	3.4g	3.4%

Synthesis and Characterization of silver nanoparticles

The synthesis of silver nanoparticles using plant leaf extracts were confirmed by color change of the mixture. Before heating, the mixture was black then it was changed to dark brown color. This color change indicated the formation of silver nanoparticles due to the conversion of Ag^{+} to Ag^{0}.

The XRD analysis was carried out to determine the crystalline nature of the particle and SEM was used to identify the shape, morphology, and size of green synthesized silver nanoparticles. The XRD pattern 00-004-0783 corresponds to the crystalline structure of silver (Ag). The pattern shows a series of peaks at specific angles (2θ) and intensities, which can be used to identify the crystal structure and orientation of the material. The first peak in the pattern appears at a 2θ angle of approximately 38.2°, which is the (111) reflection of the face-centered cubic (FCC) crystal structure of silver. The second peak appears at around 44.3°, which corresponds to the (200) reflection of the same crystal structure. The third peak appears at around 64.4°, which is the (220) reflection of the FCC structure. The other peaks in the pattern can also be indexed to the FCC structure of silver, including the (311) peak, it can be concluded that the sample is a polycrystalline silver material with an FCC crystal structure (Figure 4).

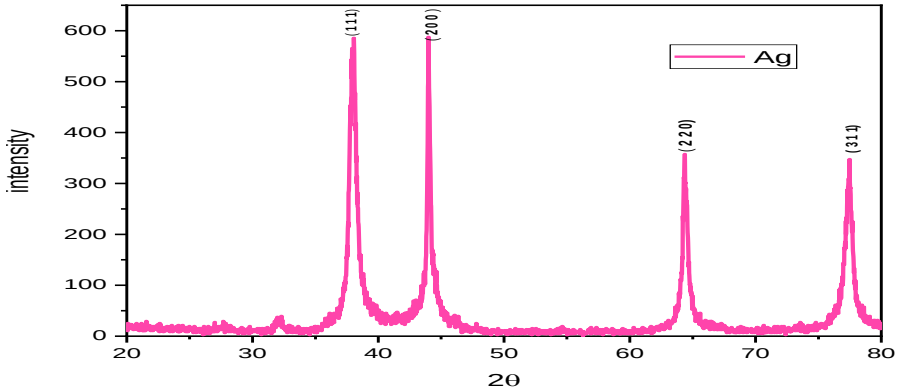


Figure 4. X-ray diffraction (XRD) analysis of synthesized silver nanoparticles

The morphology and shapes of synthesized silver nanoparticles were analyzed by scanning electron microscopy (SEM). The result is shown in Figure 5 below. The SEM analysis showed the agglomerated image of particles. This image is a nanomaterial, which means that the particles are very small, typically less than 100 nanometers in size. The image has a spherical shape. The fact that the image of nanoparticles is agglomerated, and aggregation appeared may be due to the secondary metabolites or salt concentration. There may be some sort of bonding or interaction between them, which could affect their properties. The surface of nanoparticles is dominated by small porous particles. Porous particles have small holes on their surface that can make them more reactive or better at adsorbing certain molecules.

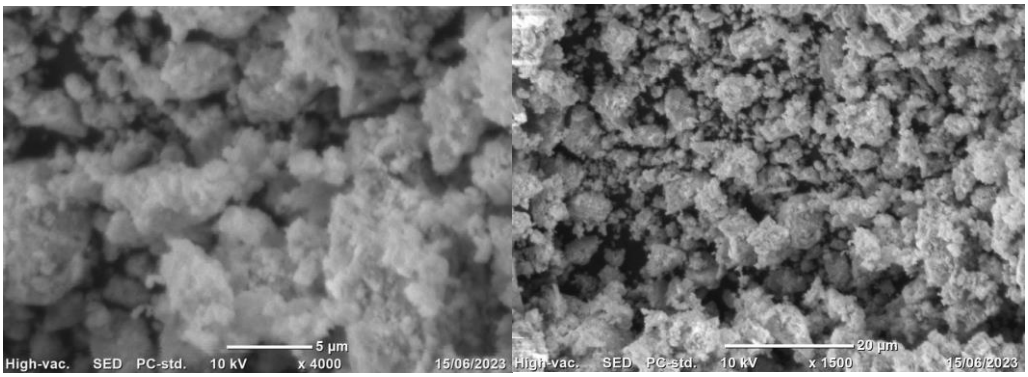


Figure 5. SEM results of synthesized silver nanoparticles

Phytochemical screening of plant extract

The phytochemical screening of *Nicotiana glauca* leaves extracts obtained by four different solvents such as methanol, distilled water, chloroform, and n-hexane was done on eight different secondary metabolites such as alkaloids, flavonoids, phenolics, saponins, steroids, terpenoids, tannins, and glycosides. The result of the test showed the presence of all tested phytochemicals. But only alkaloids were present in the methanol extract and n-hexane extract contained only flavonoids from the tested phytochemicals. Saponins, tannins and terpenoids are found in all extracts except N-hexan. Tannins and phenolic are found only in methanol and water extracts. The results are shown in Table 2 below.

Table 2. Phytochemical analysis of leaf extracts of *Nicotiana glauca*

S.no	Phytochemicals compound	Test	Extracts			
			Methanol	D/water	n-hexane	Chloroform
1	Alkaloids	Wagner test	++	+	+	++
2	Flavonoids	Alkaline reagent Test	-	-	+	-
3	phenolics	Ferric chlorides test	+	+	_	_
4	steroids	Liebermann test	-	-	++	++
5	Saponins	Foam test	++	++	-	++
6	Tannins	Lead acetate	+	+	-	-
7	Terpenoids	Salkowski's test	++	+	-	++
8	Glycosides	Glycoside test	+	+	+	-

The symbols ++, + and – refer to appreciable amounts, moderate to trace amount and absent amounts, respectively.

Evaluation of Antioxidant activities

The antioxidant activity of methanol, chloroform, n-hexane leave extracts, and synthesized silver nanoparticles was done using a DPPH scavenging assay. The results of % inhibition of ascorbic acid, crude extracts, and synthesized silver nanoparticles presented in Table 5 below. The results showed that green synthesized silver nanoparticles have the highest antioxidant activity. Next to the silver nanoparticles, methanol extract has good antioxidant activity and N-hexane extract has the lowest antioxidant activity than others (Figure 6). Generally, the antioxidant activity of the extracts and silver nanoparticles increases with increasing concentration.

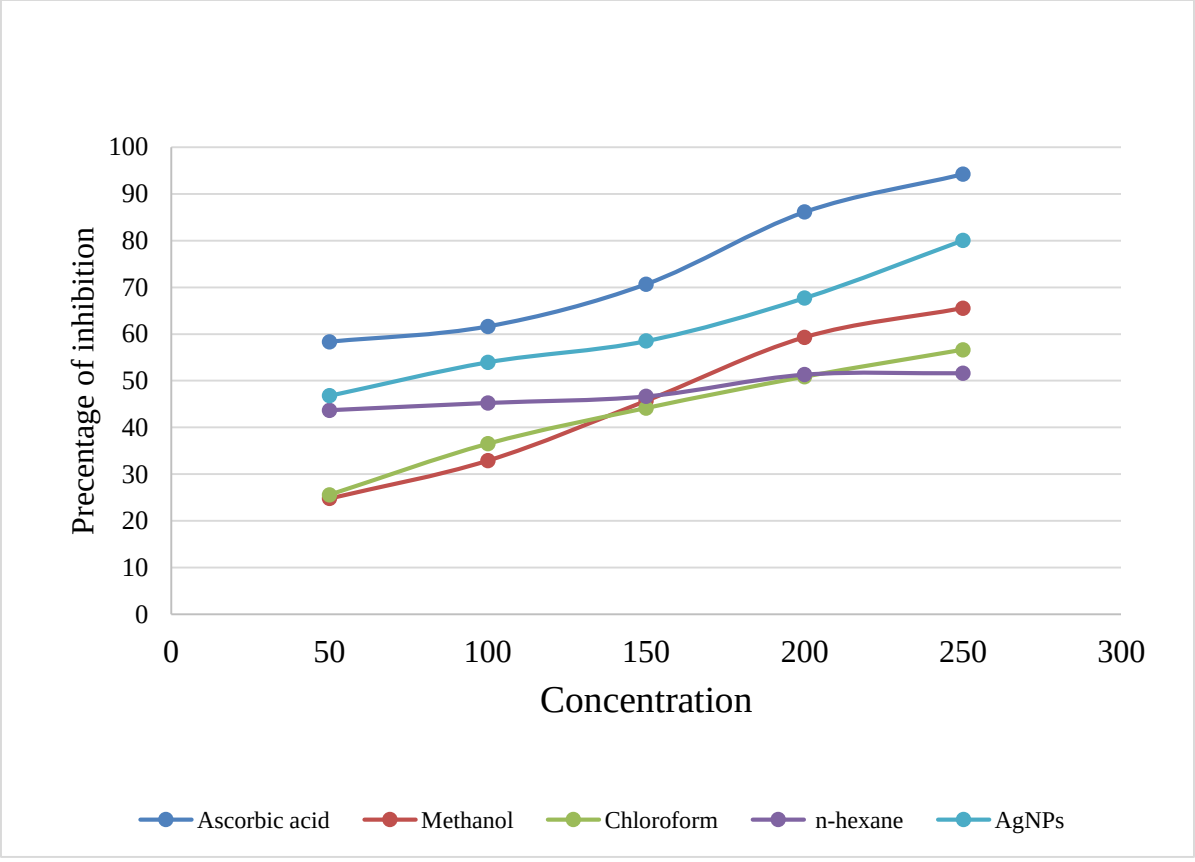


Figure 6. The percent inhibition of ascorbic acid, plant extracts, and synthesized silver nanoparticles

The IC_{50} value of the present study implied that the ascorbic acid showed a 24 µg/mL IC_{50} value. The green synthesized silver nanoparticle 78 µg/mL showed the highest activity. Methanol has an IC_{50} value of 170 µg/mL, chloroform showed 197 µg/mL, and n-hexane showed a 202 µg/mL, IC_{50}value (Table 4).

Table 4. The percent scavenging activity of plant extract and green synthesis silver nanoparticles

Concentration µg/ml	Ascorbic acid		Methanol		Chloroform		n-hexane		Silver nanoparticles	
	%Inhibition	IC_{50} µg/mL	%Inhibition	IC_{50} µg/mL	%Inhibition	IC_{50} µg/mL	%Inhibition	IC_{50} µg/mL	%Inhibition	IC_{50} µg/mL
50	58.34		24.80		25.58		43.68		46.80	
100	61.62		32.91		36.50		45.24		53.97	
150	70.67	24	45.70	170	44.14	197	46.64	202	58.50	78
200	86.11		59.28		50.85		51.32		67.70	
250	94.22		65.52		56.63		51.63		80.03	

Antimicrobial activity evaluation

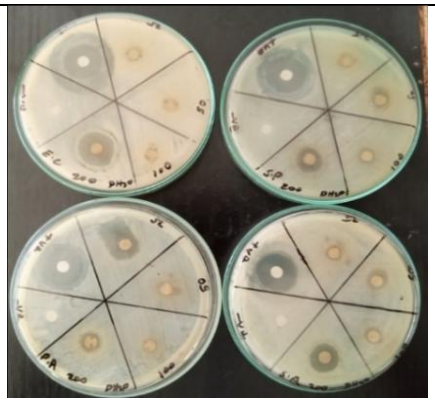
The results of antimicrobial test of the plant extracts and silver nanoparticles are given in Table 5 and Figure 8. The antibacterial activity evaluation result showed that, from the tested extracts, Methanol extract exhibited the highest antibacterial activity against *Escherichia coli* at 200mg/ml concentration followed by distilled water, Chloroform and n-hexane at concentration of 200mg/ml against *Escherichia coli, Streptococcus pyogenes* and *Staphylococcus aureus* respectively. The highest antifungal activity was exhibited by n-hexane extract against *Candida albicans.* In all the tested extracts, as concentration increases, the antibacterial activity and anti-fungal activity increases (Table 5 and Figure 8).

The synthesized silver nanoparticles also showed antibacterial and anti-fungal activity. The highest antibacterial activity and antifungal activity of the silver nanoparticles was observed on *Escherichia coli* and *Candida albicans* at 10 mg/ml respectively. Similar to the plant extracts, as concentration of the silver nanoparticles increases, the antimicrobial activity increases. When compared to the extracts, the silver nanoparticles exhibited better antimicrobial activities (Table 5 and Figure 7).

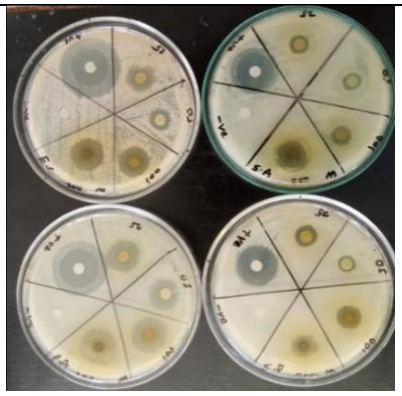
Table 5. Zone of inhibition (mean±SD) of leaf extracts and green synthesized silver nanoparticles

Extracts	Concentration mg/ml	Zones of inhibition (mean±SD) in mm				
		Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Candida albicans*
Methanol	200	15.33±1.155	14.00±1.000	14.33±0.577	14.00±2.000	12.00±1.000
	100	12.67±1.155	13.00±1.000	10.67±1.155	12.33±1.528	10.00±1.000
	50	10.00±1.000	8.67±1.155	8.33±1.528	10.00±1.000	9.00±1.000
	25	10.67±0.577	9.33±1.528	8.33±1.528	9.67±0.577	7.33±0.577
Distilled water	200	15.00±1.732	14.00±1.000	13.67±2.082	13.33±0.577	11.67±2.082
	100	10.67±2.082	11.33±1.528	11.33±1.528	10.67±1.155	10.33±1.528
	50	7.00±1.000	11.00±1.000	10.00±1.000	9.33±0.577	8.67±0.577
	25	7.67±1.155	8.33±0.577	8.33±0.577	8.00±1.000	7.67±0.577
Chloroform	200	12.67±1.155	13.67±0.577	14.00±1.000	15.00±1.000	12.33±1.528
	100	14.33±1.155	12.00±1.000	11.67±2.082	13.00±1.000	12.67±0.577
	50	12.33±1.528	11.000±1.000	9.00±1.000	10.00±2.000	12.00±1.000
	25	8.33±2.082	9.33±0.577	7.67±1.155	8.33±0.577	9.00±1.000
n-hexane	200	14.67±1.155	13.33±1.155	15.00±1.000	14.33±1.528	13.00±1.000
	100	10.67±1.528	14.33±1.528	11.00±1.000	8.67±0.577	10.33±0.577
	50	8.67±0.577	9.33±1.528	9.67±2.082	8.00±1.000	9.33±0.577
	25	8.00±1.000	8.67±1.528	7.67±1.155	7.33±0.577	8.67±0.577
Silver nanoparticles	10	16.33±1.155	15.67±1.155	15.33±1.155	15.00±1.000	15.00±1.000
	5	15.00±1.000	12.00±1.000	14.00±1.000	12.00±1.000	13.00±1.000

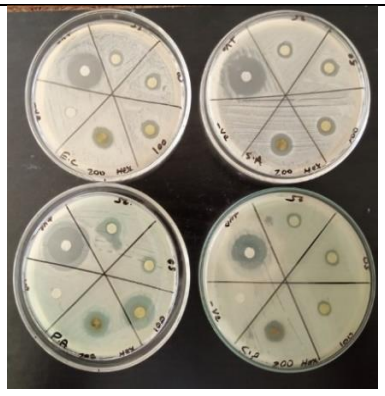
Extracts	Concentration mg/ml	Zones of inhibition (mean±SD) in mm				
		Escherichia coli	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Candida albicans***
	2.5	12.00±1.000	10.00±1.000	12.67±0.577	10.67±1.155	11.67±0.577
	1.25	10.33±1.528	9.67±0.577	10.67±0.577	10.00±1.000	9.67±0.577
Ciprofloxacin	30	19.33±0.577	21.00±1.000	23.67±1.155	19.67±0.577	-
Ketoconazole	30	-	-	-	-	20.00±1.000
DMSO	1ml	0	0	0	0	0



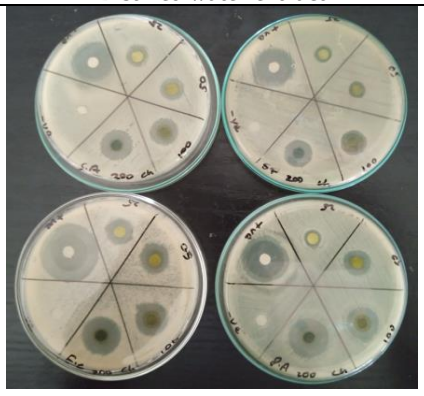
Distilled water extract



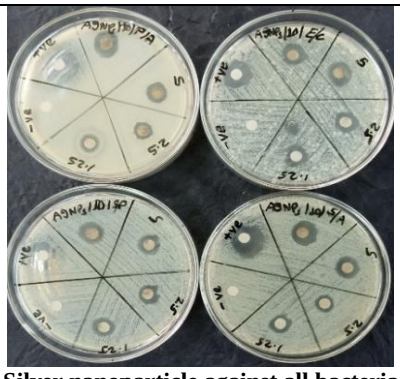
Methanol extract



n-hexane extract



Chloroform extract



Silver nanoparticle against all bacteria strains

Antifungal activity on all extracts

AgNPs against *Candida albicans*

Figure 7. Antimicrobial activities of plant extracts and green silver nanoparticles

Determination of Minimum Inhibitory Concentration

The results of minimum inhibitory concentration determined using different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml) of plant extracts and (10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, and 0.625 mg/ml) of synthesized silver nanoparticles against four bacteria strains and one fungus strain are presented in Table 6 below. The MIC of methanol and chloroform crude extract ranged from 12.5mg/ml to 50mg/ml for both bacterial and fungal species. The MIC value of Methanol and n-hexane ranged from 25 to 50mg/ml. Additionally, the MIC value of silver nanoparticles was 0.625mg/ml for *C. albican*.

Table 6. The minimum inhibitory concentration (MIC) of leaf extracts of *Nicotiana glauca*

Extracts	Minimum inhibitory concentration (in mg/ml)				
	Escherichia coli	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Candida albicans***
Methanol	25	25	25	50	12.5
Distilled water	50	50	25	25	25
Chloroform	25	25	25	25	12.5
N-hexane	25	50	50	50	50
Silver nanoparticles	1.25	1.25	2.5	2.5	0.625

Determination of Minimum Bactericidal Concentration and minimum fungicidal concentration

The MBC and MFC were determined after the determination of the MIC of bacteria and fungi, respectively. The results of MIC showed a negative microbial growth on the surface of MHA and SDA agar plates for bacteria and fungus to examine the presence or absence of microbial growth. The lowest concentration completely inhibits the bacteria and fungus considered MBC and MFC. The results of the minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) are presented in Tables 7.

Table 7. The MBC and MFC of tested bacteria and fungi

Extracts	MBC and MFC (in mg/ml)				
	Escherichia coli	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Streptococcus pyogenes***	***Candida albicans***
Methanol	100	50	100	100	50
Distilled water	100	100	50	100	100
Chloroform	100	100	50	50	50
N-hexane	100	100	100	100	100
Silver nanoparticles	10	5	5	5	5

Methanol, distilled water and chloroform crude extracts showed MBC against *S. aureus* and S. pyogenes, *E. coli, P. aeruginosa* at 50mg/ml concentration. Chloroform Showed MBC against *Streptococcus pyogenes at* 50mg/ml. The minimum bactericidal concentration against *Escherichia coli* was at concentration of 100mg/ml in all extracts. Whereas n-hexane extract inhibited all bacterial cells at 100mg/ml.

The minimum fungicidal concentration obtained by methanol and chloroform extracts were 50mg/ml. For all other extracts the MFC recorded was 100mg/ml. Moreover, Green synthesized AgNps, displayed bactericidal and fungicidal activity at low concentrations when compared with crude extracts. The synthesized silver nanoparticles inhibited killed all test bacteria and fungi at concentration of 5mg/ml except *E. coli*.

4. DISCUSSIONS

In this study, phytochemicals were extracted from the leaf of the *Nicotiana glauca* plant using different solvents. From the used solvents Methanol extract gave the best yield when compared to distilled water, chloroform, and n-hexane solvents. The chloroform showed better results when compared to the n-hexane. The yield obtained decreases as the polarity of the solvent decreases. This indicated that polar solvent gave excellent results when compared to the non-polar solvent. Other studies reported before also indicated methanol extracts were given the highest yield when compared to other solvents [33]. This might be due to the potential of methanol as a good solvent for extracting both polar and non-polar substance [34, 35].

The current study also indicated the presence of tested phytochemicals including alkaloids, terpenoids, steroids, flavonoids, tannins, saponins, phenols and glycosides in the crude extract of *Nicotiana glauca*. The phytochemical test done by [36], indicates that the water extract of *Nicotiana glauca* contains flavonoids and alkaloids but tannins, saponins, phenols, and glycosides were absent. In chloroform extract saponins are absent and glycosides are present. In n-hexane extract flavonoids were absent. The present findings disagree with some of the above results, in the distilled water extract alkaloids, tannins, phenols, glycosides, and saponins were presented but flavonoids were absent. In the chloroform extract, saponins were present, while glycosides were absent. In the n-hexane extract, flavonoids were present. The differences may arise from environmental factors such as mineral deficiencies, drought, cold, and disease [37]. The research findings reported by [38] indicated that the leaf extracts of *Nicotiana glauca* contain polyphenols, flavonoids, tannins, coumarins, terpenes, steroids, alkaloids, and quinones, which are consistent with the results of this study.

Phytochemicals that are present in the medicinal plant have a capacity for antimicrobial activity, anti-inflammatory activity, anticancer activity, antiallergic activity, antiviral activity, antimalarial activity, and so on [38]. In the present study, antioxidant activity was done by using the DPPH scavenging assay in different concentrations. In 250 µg/ml concentration: ascorbic acid, silver nanoparticles, methanol, chloroform, and n-hexane showed (94.22, 86.11, 70.67, 61.62, and 58.34) % scavenging activities, respectively. This showed that the plant extracts and the synthesized silver nanoparticles have antioxidant activities. The lowest IC_{50} value showed the highest antioxidant activity. When we compare the samples IC_{50} with ascorbic acid (24), silver nanoparticles have a 78 IC_{50} value and have the highest antioxidant activity. The present study agrees with the study done before which reported that methanol extract has shown good antioxidant activity when compared to other extracts [39]. The antioxidant properties observed may be due to the presence of phenols, terpenoids, glycosides and flavonoids in the leaf of this plant. According to studies done before, phenolic and flavonoids have a high potential to scavenge free radicals [40]. This might be due to the arrangement of their properties such as type of functional groups, their redox properties and number of hydroxyl groups which enhanced the antioxidant properties [41, 42]. The antioxidant activities exhibited by the leaf extract of Nicotiana glauca align with the previous findings which confirmed free radical scavenger activities in various efficacies across multiple plants [43 and 44].

The antimicrobial activity evaluation result indicated that all the extracts and the synthesized silver nanoparticles have antibacterial and antifungal activities. The polar

solvent extracts showed excellent antibacterial activity against gram-negative bacteria. According to this study, the leaf extracts and silver nanoparticles significantly exhibited higher antimicrobial activity by showing inhibition zone that ranged from 7.33±0.577mm to 16.33±1.155mm. Methanol extract has shown 15.33±1.155mm and distilled water 15.00±1.732mm against *Escherichia coli*. While the chloroform extract showed significant activity against *Streptococcus pyogenes* 15.00±1.00mm and the n-hexane extract showed better activity against *Staphylococcus aureus* 15.00±1.00mm. The antimicrobial activity potential of the extracts may be due to their phytochemical's contents. Previous studies confirmed that plant extracts having phytochemicals such as alkaloids, flavonoids. Phenols, saponins, steroids, tannins and terpenoids have antibacterial activities. These phytochemicals inhibit the growth of microorganisms by mechanisms such as altering the permeability of cell membrane, inactivating enzyme activity, inducing cell membrane disturbance/ anxiety, and cause bacterial cell lysis [45].

Generally, the silver nanoparticles exhibited better antimicrobial activity than the extracts which agrees with previous report [46]. The study done before also showed silver nanoparticles have the highest activity in gram-positive bacteria than gram-negative bacteria, [47], which disagrees with the result of this study. The n-hexane extracts 13.00±1.000 and silver nanoparticles 15.00±1.000 showed an inhibition zone against *Candida albicans*. Both n-hexane extract and silver nanoparticles showed better activity against *Candida albicans* than others. The present studies agree with the research done before [48], the polar solvent extract such as methanol and distilled water has excellent activity than nonpolar solvents. The potential of the antibacterial activity of silver nanoparticle is due to their potential to generate reactive oxygen species; their potential to denature proteins by bonding with sulfhydryl groups by Ag^+ ions released from AgNPs and their potential to attach on bacteria and damage them [49-50].

The present study indicated that the MIC value of methanol extract was 25 mg/ml against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus aureus* and 50 mg/ml against *Staphylococcus pyogenes*. The methanol extract of *Nicotiana glauca* showed a MIC of 12.5 mg/ml against *Candida albicans*. In distilled water extract, the MIC showed 50 mg/ml against gram-negative bacteria, 25 mg/ml against gram-positive bacteria, and 25 mg/ml against *Candida albicans*. The chloroform extract showed MIC of 25 mg/ml in all bacterial strains and 12.5 mg/ml in *Candida albicans*. The n-hexane extract showed a MIC of 25 mg/ml against *Escherichia coli* and 50 mg/ml against *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Candida albicans*. In silver nanoparticles, MIC showed 1.25 mg/ml in *Escherichia coli* and *Pseudomonas aeruginosa*, and 2.5 mg/ml in *Streptococcus aureus* and *Staphylococcus pyogenes*. The MIC was shown at 0.625 mg/ml in *Candida albicans*. The result of the study done before [51] showed that 6 mg/ml of plant extract of *Nicotiana glauca* has the potential to stop the growth of *Staphylococcus aureus* and *Escherichia coli*, this study disagrees with the present study.

The methanol extract showed MBC at the highest concentration of 100 mg/ml against *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, respectively. In *Pseudomonas aeruginosa* and *Candida albicans*, the MBC and MFC showed 50 mg/ml concentration respectively. In distilled water extract, the MBC was shown at 50 mg/ml against *Staphylococcus aureus* and 100 mg/ml against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*. The MFC value was 100 mg/ml against *Candida albicans*. In n-hexane extract, the MBC showed the highest concentration of 100 mg/ml against all bacterial strains. And the MFC also showed at 100 mg/ml. The chloroform concentration showed the MBC at 100 mg/ml against *Escherichia coli* and *Pseudomonas aeruginosa* and 50 mg/ml against *Streptococcus pyogenes* and *Staphylococcus aureus.* The MFC value of n-hexane was

50 mg/ml. The silver nanoparticle showed MBC at 5 mg/ml in *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa* and 10 mg/ml in *Escherichia coli.* The MFC was shown at 5 mg/ml.

5. CONCLUSIONS

The results of this study confirmed that the leaf extracts of the plant *Nicotiana glauca* contain alkaloids, saponins, flavonoids, steroids, terpenoids, phenolics, tannins, and glycosides. From the solvents used to extract phytochemicals, the polar solvents contain more phytochemical compounds than nonpolar solvents. The leaf extracts of this plant and silver nanoparticles synthesized using distilled water extract of *Nicotiana glauca* the highest capacity of DPPH scavenging activities. The study also concluded that the phytochemicals present in *Nicotiana glauca* had the potential of antimicrobial activity against pathogenic bacteria and fungi. The antibacterial activities of these plants were more potent in gram-negative bacteria than gram-positive bacteria. The green synthesis silver nanoparticle showed excellent antimicrobial activity than plant extracts. Generally, the plant *Nicotiana glauca* has a strong potential for antioxidant and antimicrobial activity.

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الإمكانيات المضادة للأكسدة والمضادة للميكروبات لاستخلاص أوراق نبات نيكوتيانا غلاوكامستخلصات أوراق غراهام وجزيئات الفضة النانوية المصنعة: نهج فيتوكيميائي

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ملخص

نبات نيكوتيانا غلاوكا هو نبات طبي ينتمي إلى جنس نيكوتيانا ويستخدم تقليديًا لعلاج العديد من الأمراض .تهدف هذه الدراسة إلى فحص محتوى المواد الكيميائية النباتية في مستخلصات أوراق نبات Nicotiana glauca، وتخليق جزيئات الفضة النانوية باستخدام المستخلصات، وتقييم أنشطتها المضادة للأكسدة والمضادة للميكروبات .تم جمع عينات الأوراق، وتجفيفها في الهواء، وطحنها إلى مسحوق. تم نقع مسحوق الأوراق في الماء المقطر، الميثانول، الن-هكسان، والكلوروفورم لاستخراج المواد الكيميائية النباتية .تم إجراء التحليل الكيميائي النباتي باستخدام الطرق القياسية .تمت عملية تخليق جزيئات الفضة النانوية عن طريق خلط محلول نترات الفضة بتركيز 3مللي مولار مع مستخلص النبات، وتم توصيف جزيئات الفضة النانوية الناتجة باستخدام حيود الأشعة السينية والتصوير المجهري الإلكتروني الماسح .تم تقييم الأنشطة المضادة للأكسدة للمستخلصات والجسيمات النانوية الفضية التي تم تصنيعها بواسطة اختبار DPPHللكشف عن الجذور الحرة، وتم تقييم الأنشطة المضادة للميكروبات في المختبر باستخدام طريقة انتشار الأقراص في الأجار ضد سلالات بكتيرية وفطرية مختارة .نتيجة التحليل الكيميائي النباتي أشارت إلى وجود القلويدات، والصابونينات، والفلافونويدات، والتربينويدات، والتانينات، والفينولات، والستيرويدات، والجليكوسيدات .أظهرت نتائج تقييم نشاط مضادات الأكسدة لجزيئات الفضة النانوية، ومستخلص الميثانول، ومستخلص الكلوروفورم، ومستخلص الن-هكسان أن لديها أنشطة مضادة للأكسدة جيدة بشكل ملحوظ .أظهر تحليل نشاط مضادات الأكسدة أيضًا أن نسبة التثبيط و IC50كانتا معتمدتين على الجرعة. أظهرت الجسيمات النانوية الفضية المصنعة أعلى نشاط مضاد للأكسدة بقيمة IC50تبلغ 78ميكروغرام/مل، وأعطى مستخلص الميثانول قيمة IC50 تبلغ170 ميكروغرام/مل .أظهرت نتائج تقييم الأنشطة المضادة للميكروبات أن مستخلص النبات والجسيمات النانوية الفضية المُصنَّعة لهما أنشطة مضادة للميكروبات .أعلى منطقة تثبيط تم ملاحظتها كانت 16.33±1.155مم لجزيئات الفضة النانوية المصنعة و 15.33±1.155مم لاستخراج النبات .أدنى منطقة تثبيط تم ملاحظتها كانت 9.67±0.577مم لجزيئات الفضة النانوية المصنعة و 7.33±0.577مم لاستخراج النبات .بشكل عام، تمتلك مستخلصات النباتات والجسيمات النانوية الفضية المُصنَّعة أنشطة قوية مضادة للأكسدة ومضادة للميكروبات .يجب إجراء مزيد من الدراسات على المكونات الكيميائية النباتية، والأنشطة المضادة للأكسدة، والأنشطة المضادة للميكروبات لهذه النبتة.

الكلمات الدالة: مضاد ميكروبي، مضاد أكسدة، نيكوتيانا غلاوكا، مركب نباتي، جزيئات الفضة النانوية.

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In vitro Analysis of the Anticancer and Antidiabetic Effects of *Teucrium orientale* Leaf Hydrophilic Extract Grown in Two Palestinian Geographic Areas

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ABSTRACT

Several studies have demonstrated that *Teucrium orientale* (*T. orientale*) species have therapeutic advantages, such as antioxidant, bacteriostatic, spasmolytic, and anti-inflammatory activity. This study aimed to assess the possible antidiabetic and anticancer activities of *T. orientale* leaf hydrophilic extracts collected from two distinct geographic regions in Palestine: Jerusalem and Ramallah. *T. orientale* hydrophilic extract was tested for its antidiabetic and anticancer properties on α-amylase activity and Lewis Lung Carcinoma (LLC) cells, respectively. The anticancer effect on LLC was evaluated by flow cytometry for cell proliferation and Annexin-V/propidium iodide (PI) staining for cell apoptosis. The *T. orientale* extract from Jerusalem had an IC50 of 7.43±0.84 µg/ml for inhibiting α-amylase enzyme activity, whereas the Ramallah extract had an IC50 value of 23.2±0.29 µg/ml. These values were compared to the positive control, Acarbose, which had an IC50 of 43.91±1.08 µg/ml. LLC cells were treated with one of the two extracts of *T. orientale* at different concentrations (0, 50, 100, 200, and 400 µg/ml) for 24 hours, and cell proliferation was assessed using an XTT assay. Total inhibition of LLC proliferation was achieved at 400 µg/ml in both extracts. The *T. orientale* extract from Jerusalem demonstrated a more efficient inhibitory effect at lower concentrations. Increasing concentrations of *T. orientale* (50, 100, 200, and 400 mg/ml) from the two geographic areas, Ramallah and Jerusalem, had no effect on the apoptosis rate in the control group. In contrast, elevated rates of apoptosis were observed following treatment with *T. orientale* extract in LLC cells at all tested concentrations, and this was positively associated with the late apoptosis marker Annexin-V+/PI+. Moreover, the *T. orientale* extract from Jerusalem exhibited an apoptotic rate of 90±3.4% at the highest concentration of 400 mg/ml, compared to 62.6±3.4% following treatment with the Ramallah extract. This suggests that the *T. orientale* extract from Jerusalem induced apoptosis in LLC cells more efficiently than the extract from Ramallah. The extracts derived from *T. orientale* show promising potential as a natural antidiabetic and anticancer agent, as evidenced by their ability to inhibit the α-amylase enzyme, impede the growth of LLC cells, and enhance apoptosis. Further in vivo and preclinical investigations are required to validate these effects.

Keywords: *Teucrium orientale;* α-Amylase; Lewis Lung Carcinoma; Cell proliferation; Apoptosis.

INTRODUCTION

The Mediterranean basin is home to over 300 species of *Teucrium* (Labiatae), a widespread flora genus. Traditional medicine widely uses Teucrium species as stimulants, tonics, diaphoretics, appetizers, and for treating gastric disorders and diabetes mellitus (1-3).

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Teucrium (Labiatae) is a widespread flora genus with over 300 species, most of which are found in the Mediterranean basin regions. *Teucrium* species is widely used in traditional medicine as stimulants, tonics, diaphoretics, and appetizers, as well as for stomach problems and diabetes (4). *Teucrium orientale* L. is a wildly distributed woodland and shrubland species in Mediterranean countries' semiarid, arid, and steppe areas (5). Plant decoction has a role in herbal medicine for treating many conditions, such as diabetes, wounds, fever, insomnia, neurological disorders, abdominal cramps, gastrointestinal disorders, colds, diarrhea, and hypertension (2, 6).

Phytochemical investigations stated that *T. orientale* contained many chemical compounds with significant bioactivity, including neoclerodane diterpenoids, flavonoids, iridoids, and phenolic acids, and its major constituents in the essential oil are α-pinene (25.10%), and β-caryophyllene (56.01%) (6).

Cancer and diabetes mellitus are prevalent illnesses that significantly influence global and local health. Epidemiologic research indicates that people with diabetes have a greatly increased chance of developing various cancer types. While cancer and type 2 diabetes share several risk factors, the plausible biological connections between the two illnesses remain unknown (7). Furthermore, observational investigations' data revealed a correlation between some antidiabetic treatments and an elevated cancer risk (8-10). Cancer and diabetes mellitus type 2 are diagnosed more frequently within the same individual than would be predicted by chance, even when age is considered (11, 12).

Hundreds of active components found in herbs can be used to produce pharmacological agents. It has been established that the high biochemical specificity, phytochemical diversity, and other molecular properties of natural products make them valuable as model structures for drug production. Hundreds of active components found in herbs can be used to produce pharmacological agents (3-5). In recent years, there has been a significant increase in the demand for and use of dietary supplements and drugs extracted from plants. Microbiologists, pharmacologists, botanists, and natural products chemist's combine the earth for medicinal plants to treat various illnesses (13-15).

Many investigations approved that the medicinal plants' constituents are affected by many factors, including geographical locations, types of soil, rainfall, and many other factors, so there is a differentiation between Ramallah and Jerusalem regions in the kind of soil, so we compared their biological activities (16-19). Therefore, the study aimed to evaluate the antidiabetic and anticancer properties of *T. orientale* leaf extracts from Palestine's Jerusalem and Ramallah governorates.

MATERIALS AND METHODS

Plant collection, preparation, and extraction

The aerial parts of the *T. orientale* plant were collected in June 2021 from two Palestinian regions (Jerusalem and Ramallah) during the flowering period. A pharmacognosist, Professor N. Jaradat, identified the plant using a reference book (20), at the Department of Pharmacy at An-Najah National University in the Pharmacognosy Laboratory. A specimen (Pharm-PCT-2413A and Pharm-PCT-2413B, respectively) has been deposited in the same laboratory for both samples. The fresh aerial parts of *T. orientale* were washed with clean water and dried in ordinary conditions for two weeks. Then, the dried parts were powdered and stored in special glass jars for upcoming work. The hydrophilic extract was prepared by immersing 400 g of dried *T. oriental*e samples in 4 L of boiled water for 3 days. The resulting extracts were filtered twice to ensure clarity. Then, the filtered extracts were subjected to vacuum freeze-drying using a Stellar Laboratory freeze dryer (Millrock Technology Inc., NY, USA) for 48 h. The freeze-dried extracts were then stored in a closed container at 4 °C in the refrigerator (21).

Finally, using this formula, the fractionations yields was determined:

$$\% \text{ Yield} = \frac{\text{Weight of } T.orientale \text{ extract}}{\text{weight of a dry plant}} \text{ X } 100\% \qquad \text{Equation (1)}$$

Jerusalem and Ramallah *T. orientale* hydrophilic extract yields were 7.06 and 8.15%, respectively.

The α-Amylase enzyme inhibitory method

The inhibitory assay for α-amylase was performed as described by Dastjerdi et al. (21). The antidiabetic drug acarbose was used as a positive control. Control was performed similarly, with 1 ml of 10% DMSO replacing the extracts. The following concentrations were utilized in this study: 50, 70, 100, 200, and 500 µg/ml for the plant extracts and Acarbose. The plant extract working solution (1 mg/ml) was produced by dissolving 25 mg of each plant fraction in 10% DMSO, and then a buffer solution of up to 25 ml was added. The absorbance of the tested samples was measured at 540 nm by applying the spectrophotometer by which our blank was 10 % DMSO. The inhibitory potential of α-amylase was measured by the formula shown below.

$$\text{I } (\%) = \frac{\text{ABS control} - \text{ABS extract}}{\text{ABS control}} \text{ X } 100\% \qquad \text{Equation (2)}$$

Where I (%) is the α-amylase percent inhibition.

Cell culture

The Lewis Lung Carcinoma (LLC) cancer cell line and epithelial cells isolated from normal human bronchial epithelium derived from autopsies of noncancerous individuals (BEAS-2B cells; ATCC; CRL-3588) were used as a control. Cells were cultured in high-glucose DMEM with 100 U/ml penicillin G, 10% fetal bovine serum (FBS), and 100 µg/ml streptomycin in an atmosphere of 5% CO_2 at 37 °C. LLC cell line is resistant to 1,3-bis-(2-chloroethyl)-1-nitrosourea but is sensitive to methotrexate and DMSO (4). The cells are reported to be highly tumorigenic but weakly metastatic in mice. Therefore, we speculated that when performing the α-amylase activity and the proliferation assays, the *T. orientale* plant solubilized in DMSO could have better results in the selected cell line than in other cell lines (22).

XTT cell proliferation assay and cell viability

The Cell Proliferation Kit II (XTT) was used based on a colorimetric assay to quantify cellular proliferation, viability, and cytotoxicity (23). LLC and BEAS-2B cells were seeded at approximately 1×10^3 per well in a final 100 µL DMEM medium volume in 96-well flat-bottom microtiter plates. Following overnight incubation, LCC cells were treated with both extracts of *T. orientale* (Jerusalem's or Ramallah's extracts) at concentrations of (0, 50, 100, 200, and 400 µg/ml) for 24 hours. At the end of incubation, 100 µL of XTT (Merk, 11465015001) was added to each well, and plates were then incubated at 37°C for an additional 4 h. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader (Beckman Coulter, DTX 880 Multimode Reader). We used the formula (A450–A670) of test cells/(A450–A650) of the blank to calculate the viability data. We used the Ceilometer automatic cell counter (Nexcelom Inc., USA) to verify the viability of LCC cells trypan blue dye exclusion test.

Detection of cellular apoptosis by flow cytometry

LCC and BEAS-2B cells (1×10^6 cells/well) were incubated in 6-well plates and treated with both extracts of *T. orientale* (Jerusalem's or Ramallah's extracts) at different concentrations of (0, 50, 100, 200, and 400 µg/ml) for 24 hours. For apoptosis and viability measurements, propidium-iodide (PI) and annexin V-conjugated to FITC (R&D Systems, Minneapolis, MN) were used to stain fragmented DNA and phosphatidylserine, respectively. Annexin-V (+) but propidium-iodide (-) defined early apoptosis, while annexin-V (+) but propidium-iodide (+) defined late apoptosis. Viable cells were annexin-V (-) and propidium-iodide (-). Unstained IgG isotype and FMO controls were used in every experimental setting. The analyses of cells were performed using BD LSR Fortessa cell analyzer, BD Biosciences, Mountain View, CA).

Statistical analysis

The data were presented as mean ± SD. Statistical

differences were analyzed by one-way analysis of variance (with Newman-Keuls post-tests among multiple groups) or with a two-tailed unpaired Student's t-test (for comparison between two groups). For the in vitro study, each experiment was repeated three times. The averages were then calculated and presented along with the standard deviation, and the results with $P<0.05$ were considered significant.

RESULTS

Antidiabetic α-amylase inhibitory activity

The inhibitory effect of *T. orientale* extract from Jerusalem and Ramallah governorates in Palestine on α-amylase was assessed at 50-500 µg/ml (Figure 1). Among the extractives, *T. orientale* extract from Jerusalem possessed higher α-amylase enzyme inhibitory activity. The α-amylase inhibitory activity of *T. orientale* extract from Jerusalem was 90.05±0.33% at a concentration of 500 µg/mL. In contrast, the standard Acarbose was 72.54±0.29% at the same concentration. At the same time, the *T. orientale* extract from the Ramallah region was 77.37±0.35%. The α-amylase enzyme inhibitory IC_{50} dose of *T. orientale* extract from Jerusalem was 7.43±0.84 µg/ml, while the Ramallah *T. orientale* sample IC_{50} dose was 23.2±0.29 µg/ml. In addition, the IC_{50} of Acarbose (positive control) was 43.91±1.08 µg/ml. The α-amylase enzyme inhibitory activity of *T. orientale* extracts and Acarbose were in the following order: Jerusalem extracts > Ramallah extract > Acarbose. These findings suggest that both extracts have potent α-amylase enzyme inhibitory activity and are more powerful than Acarbose (24, 25).

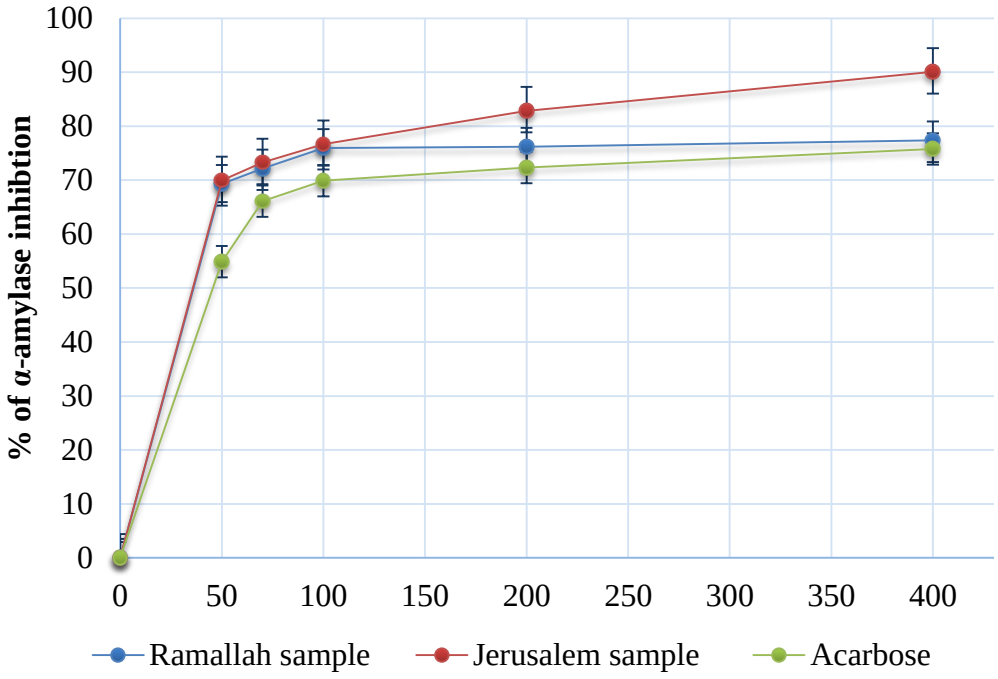

Figure 1. α-Amylase inhibitory effects of *T. orientale* extracts from Jerusalem and Ramallah regions of Palestine and Acarbose ($p< 0.05$)

***T. orientale* inhibits Lewis Lung Carcinoma (LLC) cell viability and induces apoptosis.**

To assess whether *T. orientale* could exert anticancer effects and influence lung cells viability, LCC and BEAS-2B cells were treated with one of both extracts of *T. orientale* (Jerusalem's or Ramallah's extract) at different concentrations of 0, 50, 100, 200, and 400 µg/ml for 24 h, and cell proliferation was inspected using XTT assay.

Figure 2 displays an inverse correlation obtained following increased concentrations of *T. orientale* on the LCC cell proliferation. Total inhibition of LCC proliferation was achieved at 400 µg/ml concentration in the Jerusalem (black line) and Ramallah (dash-dotted gray line) extracts. *T. orientale* of the Jerusalem extracts demonstrated a more efficient inhibitory effect at lower concentrations. *T. orientale* from both geographical areas of Ramallah and Jerusalem showed no significant effects on the viability of the lung cells of the control cells. Statistical analysis values of the t-test are presented in appendix 1.

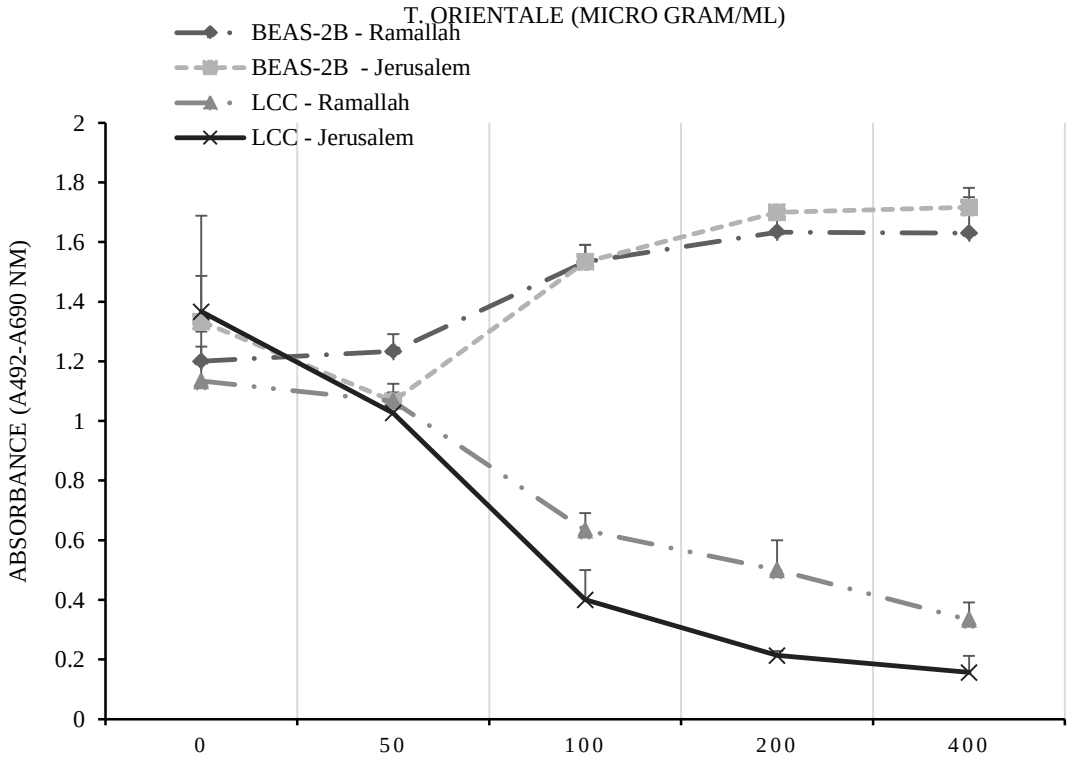

Figure 2. Effects of *T. orientale* on LCC cell proliferation

The decrease in LLC proliferation following *T. orientale* treatment was then evaluated to determine whether it was associated with changes in the apoptotic rate. Cells undergoing apoptosis exhibit cell surface phosphatidylserine (PS), which was estimated by staining with a fluorescent conjugate of Annexin-V. Necrotic cells were stained using propidium iodide (PI). Our results indicate that *T. orientale* treatment promoted late-stage apoptosis (Annexin V+/PI+) for both plant extracts (Figure 3).

The average percentage of apoptosis in all untreated *T. orientale* LLC and control cells was 9.30±2.8%. Increasing concentrations of *T. orientale* (50, 100, 200, and 400 µg/ml) from the two geographical areas, Ramallah and Jerusalem, did not affect the apoptosis rate of BEAS-2B cells (*P*=ns). In contrast, apoptosis rates significantly increased following treatment with *T. orientale* extract in LLC cells at all tested concentrations, which was positively associated with the late apoptosis marker Annexin-V+/PI+.

Moreover, the *T. orientale* extract from Jerusalem exhibited an apoptotic rate of 90±3.4% at the highest concentration of 400 µg/ml, compared to 62.6±3.4% following treatment with the Ramallah extract (*P*<0.05). This indicates that the *T. orientale* extract from Jerusalem mediates anticancer activity more efficiently than the extract from Ramallah. Statistical analysis values from the t-test are presented in Appendix 2.

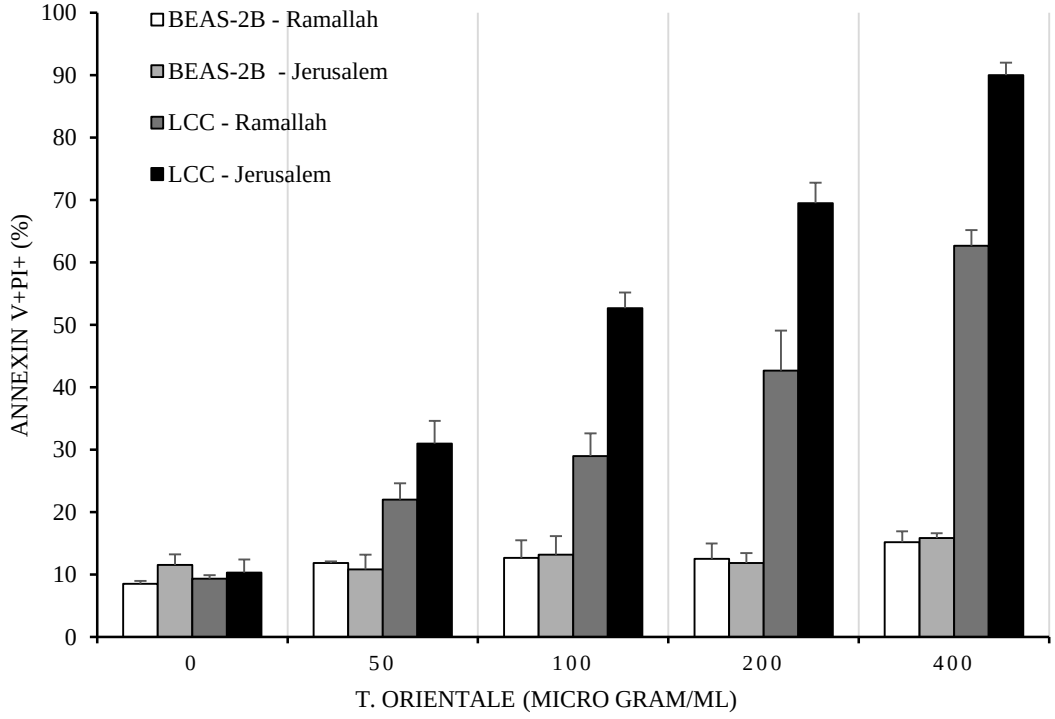

Figure 3. *T. orientale induced* apoptosis in LCC cells, measured by flow cytometry

DISCUSSION

Many bioactive compounds are derived from plants; more than 25% of available pharmaceutical forms are prepared from plant origin (26). Moreover, better therapeutic outcomes in clinical practice were reported for herbal medicine compared to chemically synthetic medications. Thus, identifying new herbal products with potential anticancer capacities has attracted notable attention in the pharmaceutical and cosmetics industries (27, 28).

Among several other Lamiaceae species, methanol extracts of *T. orientale* showed moderate to high inhibitory

effects on the enzymes α-amylase and α-glucosidase (29). In an earlier study, variable concentrations of hydroalcoholic, dichloromethane, and ethyl acetate were extracted from three *Teucrium* species and were tested for α-amylase inhibition using Acarbose as the standard inhibitor. All three studied extracts demonstrated potent inhibitory effects on the α-amylase activity. The IC_{50} for the dichloromethane, ethyl acetate, and hydroalcoholic were 22.59, 8.55, and 13.93 mg/ml, respectively (30). Previous research proved that acetone and methanol extracts of *T. orientale* had potent antioxidant activities (2, 5). Another study found that methanol extract led to a progressive increase in weight in diabetic rats and improved the associated hematological abnormalities. The authors suggested that weight might result from the fact that the extract leads to better utilization of nutrients in the diet (31). The possible role of *T. orientale* in the correction of hyperglycemia and, subsequently, in the prevention of diabetic complications was observed in another study (32). Acarbose is a hypoglycemic pharmaceutical formulation utilized to prevent and treat hyperglycemia and, in certain regions, used to treat prediabetes (33). Identifying a natural plant product with an antidiabetic effect that exceeds the potent effect of Acarbose is of great importance. Other plant species with in vitro anti-α-amylase and anti-α-glucosidase effects including *Achillea santolina*, *Coriandrum sativum*, *Teucrium barbeyanum***,** and *Teucrium polium* were also previously identified (34-36). Shown in Table 1.

Table 1. Amylase IC_{50} values of different plant extracts

Plant	IC_{50} of α- amylase inhibitory activity	Reference
Ethanol and hexane extracts of *Phyllanthus amarus*	36.05±4.01 µg/ml and 48.92±3.43 µg/ml, respectively	(37)
Ethyl acetate extract of *Phlomis bruguieri*	1.9 µg/ml	(38)
Teucrium polium	3.63 mg/ml	(39)
Teucrium oliverianum	3.86 mg/ml	(39)
Ethanol extracts of *Allium akaka*, *Allium sativum*, *Allium porrum*, and *Allium cepa*	16.74, 17.95, 15.73, and 16.36 mg/ml, respectively	(40)
Pomegranate leaves extract	43.24 µg/ml	(41)
Ethanolic extract of *Andrographis paniculata*	50.9±0.17 mg/ml	(42)
DMSO plant extract of *Teucrium Orientale*	13.93 mg/ml	(15)

One of the antidiabetic treatment protocols is to suppress carbohydrate metabolism in the gastrointestinal tract by inhibiting the action of enzymes like α-amylase involved in carbohydrate metabolism. Medicinal herbs delay glucose absorption by inhibiting the carbohydrate hydrolyzing enzymes such as pancreatic α-amylase. When this enzyme is inhibited, it slows down carbohydrate digestion. It extends the carbohydrate digestion period, reducing the rate of glucose absorption and, hence, reducing the postprandial plasma glucose level (43-45). Numerous studies demonstrated the inhibitory effects of the α-amylase enzyme of traditional medicinal herbs or their isolated products (37, 46, 47).

Most of the anticancer compounds were derived from natural sources (48). It is identified in the literature that more than 290 essential oils and non-volatile extracts are found in various species within the *Teucrium* taxa (3). *Teucrium* species were found to be rich in polyphenolic compounds, which might directly contribute to their antiproliferative and proapoptotic activity. Antioxidant, antiproliferative, and apoptotic effects of several *Teucrium* species, such as *T. chamaedrys*, *T. arduini*, and *T.*

montanum were previously identified (49). *T. persicum* showed a potent anti-tumor effect in a highly invasive prostate cancer cell line (50). *T. chamaedrys* methanol extract modulates apoptosis and biotransformation in colorectal carcinoma cells, causing apoptosis of SW480 cells, without affecting normal HaCaT keratinocytes (51). *Teucrium orientale* extract is found to be rich in chemical ccompounds such as aldehydes, hydrocarbons, monoterpene hydrocarbons, and other volatile components (52). However, the hydophilc leaf extract of *T. orientale ,* the anticancer effect of *T. orientale*, and the apoptotic potential of *T. oriental* was not previously studied to the best of our knowledge. The current study revealed the anti-diabetic, antiproliferative and apoptotic effects of *T. orientale* hydrophilic leaf extract on an in vitro model.

Besides habitat-related anatomic and morphological adaptation of *Teucrium* (Lamiaceae) species, total phenol quantity and essential oils identity were varied in different *Teucrium* species based on the geological substrate and habitat conditions such as soil mineral and water content (53-58). In the current study, the aerial parts of *T. orientale* from two regions in Palestine, Jerusalem and Ramallah were isolated and tested for their antidiabetic and anticancer effects, which are variable based on the geographical area. Although there are geographical similarities between the two regions regarding the "terra rossa" soil, Jerusalem's soil is more clay than Ramallah's. The characteristics of clay soils include that they are heavy, rich in nutrients, wet in winter, and dry in summer. Moreover, the temperature difference varies between the two cities; Jerusalem is 2 to 3 degrees less than Ramallah. Total annual rainfall in Ramallah is>700mm, while in Jerusalem ranges from 300-500 mm. These differences could contribute to the superior effects of *T. orientale* of Jerusalem concerning their antidiabetic and anticancer properties (59).

Presently, there are several antidiabetic drugs used for treating and managing diabetes. Several modes of action were proposed, including inhibiting the enzymes α-amylase, lipase, α-glucosidase, and DPP-IV. Our results indicated that the extract inhibited α-amylase even better than the Acarbose drug. Moreover, *T. orientale* caused cell death via apoptosis/necrosis of LCC in favor of the Jerusalem extract. Data was associated with reduced proliferation, indicating the ability of the *T. orientale* to prepare the cells to shift to necrosis.

CONCLUSION

The current study on the leaf hydrophilic extracts of T. orientale aerial parts from two Palestinian regions demonstrated potential α-amylase inhibitory activity compared to the antidiabetic drug Acarbose. Additionally, both T. orientale extracts inhibited LLC cell proliferation and induced cell death via apoptosis/necrosis, with the Jerusalem extract showing greater efficacy. Furthermore, this study highlights opportunities for future research in the search for novel, effective, natural antidiabetic and anticancer therapies. Further in vivo studies are needed to confirm these findings.

Appendix 1: The t-test analysis of the four tested groups of XTT assay

TTEST					
	0	**50**	**100**	**200**	**400**
AB	0.13728831	0.01205506	0.5	0.06003945	0.16830581
AC	0.2458835	0.00552825	2.2173E-05	3.5105E-05	3.7449E-05
AD	0.21975577	0.00269917	3.5105E-05	1.0388E-06	2.1848E-05
BC	0.072352	0.5	2.2173E-05	1.6146E-05	5.1662E-06
BD	0.5	0.00806504	0.03312991	0.06875769	0.05584542
CD	0.43949819	0.17432057	3.5105E-05	7.5794E-09	2.9524E-06

A=BEAS-2B – Ramallah B=BEAS-2B - Jerusalem
C=LCC – Ramallah D=LCC - Jerusalem

Appendix 2: The t-test analysis of the four tested groups of apoptosis assay

TTEST					
	0	**50**	**100**	**200**	**400**
AB	0.01976872	0.25357875	0.42232288	0.35871418	0.28950771
AC	0.06588878	0.0013528	0.00176113	0.00081438	5.7528E-06
AD	0.10608033	0.00039131	2.6529E-05	9.0208E-06	5.3181E-07
BC	0.04825265	0.00274891	0.00214858	0.00064378	3.2917E-06
BD	0.01673587	0.02388436	0.07745599	0.03815193	0.05527494
CD	0.23522311	0.00063056	3.1842E-05	5.3113E-06	2.31E-07

A=BEAS-2B – Ramallah B=BEAS-2B - Jerusalem
C=LCC – Ramallah D=LCC - Jerusalem

Data Availability

All data supporting the findings of the study are included in the manuscript.

Authors contribution

The authors performed all parts of this study.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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تحليل مختبري للتأثيرات المضادة للسرطان والسكري لمستخلص أوراق Teucrium orientale المحب للماء المزروع في منطقتين جغرافيتين في فلسطين

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ملخص

أظهرت العديد من الدراسات أن أنواع Teucrium orientale تمتلك خصائص علاجية مهمة، بما في ذلك النشاط المضاد للأكسدة، والمضاد للجراثيم، والمضاد للالتهابات، إضافةً إلى تأثيرها المزيل للتشنجات. تهدف هذه الدراسة إلى تقييم الأنشطة المحتملة المضادة لمرض السكري والسرطان لمستخلصات أوراق Teucrium orientale المحبة للماء، التي جُمعت من منطقتين جغرافيتين في فلسطين: القدس ورام الله. تم اختبار المستخلص المائي للنبات لتحديد تأثيره المضاد لمرض السكري عبر قياس تثبيط نشاط إنزيم α-amylase، بينما تم تقييم تأثيره المضاد للسرطان على خلايا سرطان الرئة Lewis Lung (LLC)باستخدام قياس التدفق الخلوي لفحص تكاثر الخلايا، بالإضافة إلى تلطيخ Annexin-V / بروبيديوم يوديد (PI) للكشف عن موت الخلايا المبرمج. أظهر مستخلص Teucrium orientale من القدس قدرة تثبيطية قوية لإنزيمα-amylase بقيمة IC50 تبلغ 7.43 ± 0.84 ميكروغرام/مل، مقارنة بـ IC50 لمستخلص رام الله الذي بلغ 23.2 ± 0.29 ميكروغرام/مل. كانت هذه القيم أفضل من الشاهد الإيجابي Acarbose، الذي سجل IC50 =43.91 ± 1.08ميكروغرام/مل. في اختبار التأثير المضاد للسرطان، تم تعريض خلايا LLC لتركيزات مختلفة من مستخلصي T. orientale (0، 50، 100، 200، و400 ميكروغرام/مل) لمدة 24 ساعة، وتم قياس تكاثر الخلايا باستخدام اختبار XTT لوحظ التثبيط الكامل لنمو الخلايا عند 400 ميكروغرام/مل في كلا المستخلصين، إلا أن مستخلص القدس أظهر كفاءة أكبر في التثبيط عند التركيزات المنخفضة. من ناحية أخرى، لم تؤثر الزيادة في تراكيز المستخلصات من المنطقتين (50، 100، 200، و400 ميكروغرام/مل) على معدل موت الخلايا المبرمج في العينات الضابطة، بينما أدى العلاج بمستخلص Teucrium orientale إلى ارتفاع ملحوظ في معدل موت الخلايا المبرمج في خلايا LLC بجميع التراكيز المختبرة. وكان هذا التأثير مرتبطًا بزيادة علامة موت الخلايا المبرمج المتأخر Annexin-V+/PI- علاوةً على ذلك، أظهر مستخلص القدس معدل موت خلايا مبرمج بلغ 90 ± 3.4% عند أعلى تركيز (400 ميكروغرام/مل)، مقارنة بـ 62.6 ± 3.4% لمستخلص رام الله، مما يشير إلى كفاءته الأعلى في تحفيز موت الخلايا المبرمج لخلايا LLC بناءً على هذه النتائج، تمتلك مستخلصات Teucrium orientale إمكانات واعدة كعوامل طبيعية مضادة لمرض السكري والسرطان، نظرًا لقدرتها على تثبيط إنزيم α-amylase، وتقليل تكاثر الخلايا السرطانية، وتعزيز موتها المبرمج. إلا أن هناك حاجة إلى مزيد من الدراسات في النماذج الحية والتجارب قبل السريرية للتحقق من هذه التأثيرات.

الكلمات الدالة: Teucrium orientale؛ ألفا الأميليز؛ سرطان الرئة لويس؛ تكاثر الخلايا؛ موت الخلايا المبرمج.

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Caregiver Views and Practices Regarding Children's Antibiotic Suspensions in Lebanon

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ABSTRACT

Lebanon grapples with the pressing challenge of pediatric antibiotic misuse. To tackle this issue, we conducted a cross-sectional study in Beirut aimed at assessing caregivers' knowledge and practices regarding children's antibiotic suspensions. A total of 113 caregivers were interviewed at pharmacies to evaluate their comprehension of medication instructions, dosing accuracy, storage/disposal practices, and satisfaction with pharmacist counseling. Results indicated that while 67.3 % of caregivers demonstrated a clear understanding of medication instructions and 63.7 % followed proper reconstitution, concerning practices surfaced. Notably, 71.7 % of caregivers stored leftover suspensions in kitchens. However, 56.6 % correctly disposed of them. Widespread use of cold mineral water as a diluent (66.4 %) contradicts guidelines. Pharmacists played a crucial role, with 69.1 % of caregivers receiving counseling, yet averaging only 3.7 minutes, suggesting room for improvement. Significant correlations emerged between accurate understanding and proper reconstitution ($p < 0.001$). These findings highlight the need for clearer instructions for caregivers, educational initiatives on appropriate storage and disposal, and enhanced training for pharmacists. Addressing these aspects can improve health outcomes in Lebanon and contribute to the global fight against antibiotic resistance.

Keywords: Antibiotic suspension use; Caregivers; Knowledge and practices; Drug administration; Optimization; Pharmacist counseling

INTRODUCTION

Antimicrobial resistance (AMR) has been deemed a global health emergency by the World Health Organization (WHO) and the United Nations (UN) General Assembly (1). It poses a threat to the life-saving power of antibiotics, causing millions of deaths annually, particularly in low- and middle-income countries (2,3). Factors contributing to this issue include misuse, limited diagnostic methods, and the presence of antibiotics in the environment (4). Addressing this requires improved infection prevention, increased diagnostic accessibility, and antibiotic stewardship (5).

In Lebanon, antibiotic misuse is widespread, ranging from 42 % to 63 %, with 22 % self-prescribing and 7 % following non-medical advice, straining public healthcare systems and fueling the emergence of AMR (6,7). This rising problem of AMR poses a critical challenge in the treatment of common infections, particularly for children with underdeveloped immune systems and those living in poverty (8,9). Optimizing antibiotic use, especially for children, is crucial to reduce treatment failures, minimize side effects, and promote better long-term health outcomes (10). Similarly, a study in Jordan by Ayyash et al. revealed

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that, despite a good level of knowledge regarding antibiotics, misconceptions and improper use persisted, emphasizing the need for focused educational efforts to address these practices (11). Likewise, research by Sonji et al. in Lebanon highlighted a knowledge-practice gap among pharmacy students regarding food labels, further underscoring the importance of addressing such gaps through targeted education (12).

The COVID-19 pandemic in Lebanon has led to increased antibiotic use, emphasizing the urgency of awareness and control measures (13). While urban residents in Lebanon generally have a good understanding of antibiotics, due to better access to healthcare facilities and educational resources, Beirut faces particular challenges due to misconceptions, which necessitate educational campaigns to curb misuse and combat AMR (14,15).

There is a significant research gap regarding the appropriate usage and teaching of antibiotic suspensions. Recent research indicates that caregivers often lack understanding of the correct amount and duration of antibiotic administration for children. For example, some caregivers may discontinue the antibiotic course once the child's symptoms improve, which can lead to inadequate treatment and potentially contribute to AMR (16–18). Additionally, there is a lack of comprehensive information on the proper instruments and practices for administering antibiotic suspensions to children, leading to either ineffective therapy or unnecessary exposure to antibiotics (19,20). Improper storage and disposal methods can reduce antibiotic effectiveness and contribute to environmental pollution (21,22). Furthermore, the impact of antibiotic suspensions on the development of AMR has not been adequately studied (23,24).

This study tackles pediatric antibiotic misuse in Beirut, Lebanon, where knowledge gaps exist regarding caregiver practices for antibiotic suspensions. To bridge this gap and optimize antibiotic use, we assessed caregiver knowledge and practices concerning reconstitution, storage, dosing, and disposal. We also evaluated their understanding of medication instructions, storage conditions, and disposal methods for antibiotic suspensions, and investigated the role of pharmacist counseling on caregiver knowledge and practices related to antibiotic use. This comprehensive approach aims to inform interventions that will ensure optimal health outcomes for children in Beirut.

METHODOLOGY

Study Design and Data Collection

This study utilized a cross-sectional design to evaluate caregiver knowledge and practices concerning antibiotic suspensions in children. Data was collected through face-to-face questionnaires administered to 113 customers at community pharmacies in Beirut, Lebanon. A convenience sampling method was used to recruit participants who were visiting pharmacies to purchase or pick up antibiotic suspensions for children. Data collection occurred between November 2022 and June 2023.

Inclusion/Exclusion Criteria

The selection of research participants was based on predetermined inclusion and exclusion criteria. Eligible participants were individuals who were purchasing an antibiotic suspension for a child under 12 years old and were at least 18 years old. Additionally, participants needed to demonstrate their ability to comprehend the questions and respond in either Arabic or English. This requirement was put in place to ensure consistent data collection that could be accessed by a wider audience. To accommodate the participants' preferred language, the questionnaire was administered in both Arabic and English. Out of the 113 people surveyed, 48 responded in Arabic, and 65 responded in English. Conversely, the study excluded individuals who were unable to understand the questionnaire due to cognitive or language limitations.

Sample Size

This study employed a convenience sampling method for participant recruitment. A power analysis was conducted before data collection to determine the necessary sample size to detect a moderate effect size with

a high level of statistical power. The analysis targeted an effect size of 0.5 for the primary outcome measure (patient satisfaction) and a desired alpha level of 0.05. This indicated a sample size of 86 participants would be required to achieve a power level of 0.90. A total of 113 caregivers of children in Beirut, Lebanon, participated in the study, exceeding the target sample size and further strengthening the generalizability of the findings.

Ethical Considerations

This study was approved by the Institutional Review Board of Lebanese International University (LIU), and written informed consent was obtained from all participants before data collection.

Questionnaire Design

The study utilized a face-to-face questionnaire designed to assess various aspects of caregivers' knowledge and practices regarding antibiotic suspensions for children with a particular focus on understanding factors affecting satisfaction with pharmacist counseling. The questionnaire was developed based on a review of previous studies (25,26) and covered various domains. These domains included demographic information (age, gender, education level, child relationship) to understand the participant pool. Additionally, it assessed caregivers' ability to read and understand pamphlet instructions for medication use. Furthermore, the questionnaire explored practices related to reconstituting, administering, and storing antibiotic suspensions to determine adherence to proper procedures. Finally, it included questions to assess caregiver satisfaction with pharmacist counseling, such as the time spent discussing medications, the adequacy of information provided, and caregivers' perceptions of the pharmacist's attentiveness and communication skills**.** By analyzing responses to these questions, we aimed to identify which aspects of pharmacist counseling (e.g., time spent, information adequacy, communication skills) contribute most to caregiver satisfaction. A 5-point Likert scale was used to measure satisfaction with different aspects of the counseling received by caregivers, ranging from 1 (strongly disagree/dissatisfied) to 5 (strongly agree/satisfied). The mean score of all responses was then calculated to obtain an overall satisfaction score.

To ensure accessibility for Beirut's diverse population, the questionnaire was translated into Arabic by two bilingual translators. Discrepancies were resolved through consensus meetings and a third bilingual translator performed a back-translation. The research team compared the back-translation to the original English version to ensure accuracy.

Data Collection

Data was collected by a single trained research assistant who conducted all interviews with the participants. This approach was chosen to maintain consistency in data collection procedures and to minimize variability in how questions were asked and responses were recorded.

Validity and Reliability

The questionnaire was assessed for content validity by a panel of experts in pharmacy and pediatrics. The internal consistency of the questionnaire was evaluated using Cronbach's alpha coefficient, which was deemed acceptable (>0.8) for all domains.

Assessment of Interviewers' Practices

To evaluate the interviewers' practices, their actions were compared with the instructions on the drug boxes and package inserts for each antibiotic used. This was done by observing the interviews and reviewing the completed questionnaires. Any discrepancies were noted and addressed with the interviewers to ensure consistency.

Pharmacist counseling was assessed by asking participants to recall the information they received from the pharmacist about the antibiotic suspension. Patient satisfaction was measured using a 5-point Likert scale for various aspects of the care they received.

Statistical Analysis

Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS) version 26.0 software. Descriptive statistics (means, standard

deviations, frequencies, and percentages) were used to summarize the data. One-sample t-tests were employed to assess whether caregivers' mean satisfaction scores significantly differed from a neutral satisfaction point (3.0) for various aspects of pharmacist interactions. Correlation analysis was used to examine the relationships among different satisfaction measures. The level of significance was set at $p < 0.05$.

RESULTS

The participants in this study (n=113) were primarily females (76.1 %), representing mothers caring for their children (62.8 %). Ages ranged from 20 to 41 years, with the largest group belonging to the more than 41 age (33.6 %). Educational attainment showed a high level of qualification, with over half (54.9 %) holding university degrees. Employment status indicated that most participants (70.8 %) were currently employed. Family size primarily consisted of one to three children (86.7 %), with the majority of children falling above the age of five (58.4 %) (Table 1).

Table 1. Socio-demographic characteristics of caregivers

Characteristics	Frequency(Percentage)
Gender	
Male	27(23.9 %)
Female	86(76.1 %)
Relation to the patient	
Father	23(20.4 %)
Mother	71(62.8 %)
Brother/sister	12(10.6 %)
Other	7(6.2 %)
Age	
Less than 20 yo	7(6.2 %)
20 to 30 yo	31(27.4 %)
31 to 40 yo	37(32.7 %)
More than 41 yo	38(33.6 %)
Education level	
School Education	51(45.1 %)
University Education	62(54.9 %)
Work	
Yes	80(70.8 %)
No	33(29.2 %)
Number of children	
1	27(23.9 %)
2	50(44.2 %)
3	21(18.6 %)
More than 3	15(13.3 %)
Child’s age (years old)	
Less than 1 yo	8(7.1 %)
1 to 3 yo	16(14.2 %)
3 to 5 yo	23(20.4 %)
More than 5 yo	66(58.4 %)

Table 2 provides a comprehensive overview of recommended reconstitution methods, water choices, and storage conditions. Only two-thirds (63.7 %) followed the recommended gradual addition technique, which involves

adding water in stages with shaking. Practices such as direct addition (15.9 %) and halfway split (20.4 %) were also observed. Cold mineral water emerged as the preferred diluent for most participants (66.4 %), followed by hot mineral water (14.2 %) and boiled and cooled mineral water (10.6 %) (Table 2).

Kitchen cabinets (71.7 %) were the most common location chosen for storing antibiotic powder, followed by living rooms (28.3 %) (Table 2). Participants' estimates for storage duration varied. Some estimated a two to three-year lifespan (33.6 %), while others estimated shorter durations (12.4 % believing less than a week) or longer lifespans (16.8 % assuming one year). Reconstituted suspensions were primarily stored in the refrigerator (65.5 %) (Table 2). Beliefs about reconstituted suspension lifespan also varied. Over half (56.6 %) believed it lasted seven days, but some had different estimations: 15.9 % thought two to three days, and 7.1 % believed it lasted more than a month.

Table 2. Methods, Water Choices, and Storage Conditions for Powder & Reconstituted Suspension

Practice	Frequency(Percentage)	Recommended
Reconstitution Method		
Gradual Addition (Tap bottle to loosen the powder, add half the volume of water, shake well, add remaining water, shake well)	72(63.7 %)	Yes
Direct Addition (Add all water, shake well)	18(15.9 %)	No
Halfway Split (add half the volume of water, shake well, add remaining water, dispense without shaking)	23(20.4 %)	No
Water Choices		
Boiled and cooled tap water	4(3.5 %)	Yes
Boiled and cooled mineral water	12(10.6 %)	No
Cold tap water	3(2.7 %)	No
Cold mineral water	75(66.4 %)	No
Distilled water	3(2.7 %)	Yes
Hot mineral water	16(14.2 %)	No
Storage Locations of Antibiotic Powder		
Kitchen cabinets	81(71.7 %)	No
Living rooms	32(28.3 %)	No
Storage Temperature of Powder		
25ºC	80(70.8 %)	Yes
37ºC	8(7.1 %)	No
18ºC	25(22.1 %)	No
Shelf Life of Powder		
Less than 1 week	14(12.4 %)	No
1 week	33(29.2 %)	No
1 year	19(16.8 %)	No
2 to 3 years	38(33.6 %)	Yes
More than 3 years	9(8 %)	No
Storage Temperature of Reconstituted Powder		
Refrigerator	74(65.5 %)	Yes
Room temperature	39(34.5 %)	No
Shelf Life of Reconstituted Powder		
2 to 3 days	18(15.9 %)	No
7 days	64(56.6 %)	Yes
10 to 14 days	23(20.4 %)	No
More than a month	8(7.1 %)	No

Table 3 presents caregiver practices in administering antibiotic suspensions, showing frequencies and percentages related to various aspects of administration and dosing tools. The majority of caregivers demonstrated consistent behaviors in reading instructions (67.3 %) and understanding provided information (67.3 %). When it came to selecting dosing tools, syringes were the preferred choice, used by 34.5 % of participants. However, a significant number of caregivers also mentioned using medicinal spoons (30.1 %), household teaspoons (17.7 %), and household cups (4.4 %) for administering antibiotic suspensions. In terms of dosing practices and hygiene, almost all caregivers (96.5 %) reported following recommended hygiene practices for dosing tools, such as washing hands and following cleaning instructions.

Table 3: Caregiver Practices in Antibiotic Suspension Administration

	Frequency(Percentage)
Category	
Reading instructions	76(67.3 %)
Understanding provided info	76(67.3 %)
Choice of dosing tools	
Syringes	39(34.5 %)
Medicinal spoons	34(30.1 %)
Medicinal cups	15(13.3 %)
Household teaspoons	20(17.7 %)
Household cups	5(4.4 %)
Dosing Practices	
Dosing tools hygiene*	
Yes	109(96.5 %)
No	4(3.5 %)

* Washing hands, following cleaning instructions

A one-sample t-test was employed to assess whether caregivers' mean satisfaction scores significantly differed from a neutral satisfaction point. Caregivers reported a mean satisfaction score of 3.7 for the time pharmacists dedicated to discussing medications, significantly higher than the neutral point of 3.0 (p=0.001). Similarly, the adequacy of counseling yielded a mean score of 3.66, also significantly higher than neutral (p=0.002). These results indicate that both time spent and counseling adequacy are key factors enhancing caregiver satisfaction. Correlation analysis supported these findings, with strong positive correlations for time spent (coefficient=0.62, p=0.001) and counseling adequacy (coefficient=0.51, p=0.001). In contrast, moderate correlations were found for medical history inquiry (coefficient=0.45) and antibiotic follow-up (coefficient=0.38), with non-significant t-test results (p=0.123 and p=0.210, respectively). This study revealed positive trends, with a high percentage of participants receiving counseling on crucial topics: 69.1 % on side effects, 75.5 % on administration frequency, and 66.4 % on reconstitution techniques. Notably, 90.5 % of caregivers learned about the importance of shaking the medication, a vital step for proper medication dispersal.

DISCUSSION

The proper use of antibiotics is crucial for combating AMR, a significant global health threat, particularly in pediatric populations (27). Antibiotic reconstitution practices, particularly the seemingly simple step of tapping the bottle to loosen the powder, require greater attention than previously recognized (28). While a large majority (63.7 %) followed the recommended "gradual addition" method, a considerable number of caregivers (36.3 %) used potentially inaccurate methods. Studies by Kumarasinghe et al. and Parihar et al. found even higher percentages (46.3 % and 65.0 %, respectively) of participants who did not shake the reconstituted suspension before each dose (28,29). Failing to shake the suspension can lead to uneven distribution of medication, potentially causing inaccurate dosing and reduced effectiveness (30). Deviation from the recommended reconstitution method was observed, with some caregivers adding all the water at once (15.9 %) or half at a time (20.4 %), raising concerns about accurate dosing. This finding is consistent with both Al-Ramahi et al. (deviations in adding water) and Olorukooba et al. (issues with reconstitution

techniques) (31,32). Educational initiatives focusing on reconstitution techniques and suspension shaking are crucial for caregivers to ensure safe and effective antibiotic use in children (23).

A concerning trend was identified in the water choices used for reconstituting antibiotic suspensions. Only a small percentage (3.5 %) used the recommended boiled and cooled tap water or distilled water (2.7 %), while the majority (66.4 %) adopted cold mineral water. This deviates from recommendations that advocate for distilled or boiled and cooled tap water (33). The use of water sources like cold tap water (2.7 %) and hot mineral water (14.2 %) is particularly worrying. Studies have shown that unboiled tap water can contain bacteria, and the composition of mineral water can impact the effectiveness of some antibiotics (34,35). Our findings align with Aika et al., who discovered that a majority of caregivers (71.7 %) believe that hot or warm water can be used to reconstitute antibiotics (26). However, they contrast the findings of Al-Ramahi et al. who reported that 75.5 % of mothers were using boiled and cooled tap water in line with recommendations (31). Olorukooba et al. also observed that 59.4 % of caregivers use bottled water, emphasizing the importance of educating individuals on best practices in different regions (32).

A positive finding is that the majority of caregivers stored the powder at the recommended temperature of 25°C (70.8 %). However, improper storage locations like kitchen cabinets (71.7 %) and living rooms (28.3 %) were concerning. Similar findings regarding improper storage locations were reported by Alsabra et al. and contrast with the results of Al-Ramahi et al. (18.0 % in the kitchen and 12.0 % in dining rooms) (31,36). Exposure to heat, light, or moisture in these locations can degrade the medication (22,37). A significant portion of caregivers underestimated the shelf life of both the powder and reconstituted suspension. This aligns with the findings of Hu et al. in the uneducated group (38). In the study conducted by Al-Ramahi et al., 56.5 % of caregivers stored reconstituted suspensions in the refrigerator (31). Our study identified that 43.4 % of caregivers held misconceptions regarding the shelf life of antibiotic suspensions. This finding aligns with Olorukooba et al., where over 60 % of caregivers did not keep reconstituted suspensions in the refrigerator (32). Reconstituted dry powder suspensions typically remain stable in the refrigerator for a specified period, usually seven to ten days (39). Misunderstanding shelf life can lead to the use of expired medication, potentially reducing its effectiveness (40).

It is encouraging to note that a high percentage of caregivers (67.3 %) reported reading medication instructions and understanding the information provided. This aligns with the findings of Qu et al., where more than 90 % of parents believed that reading instructions carefully before using antibiotics was necessary (41). Both pharmacists and doctors provided guidance (55.4 % and 44.6 %, respectively), but it is essential to encourage patients to actively seek and comprehend information from both leaflets and healthcare professionals for optimal medication management. These findings are consistent with Al-Ramahi et al., where 86.8 % of mothers reported reading instructions, with pharmacists and doctors being the primary sources of advice (31). Aika et al. also found that caregivers were open to education on antibiotic use, resulting in a significant improvement in knowledge and practices after an educational session (26). Similarly, Tadesse et al. reported that 54.3 % of patients were satisfied with pharmacy services (42). These findings are contrasted by a study conducted by Machongo et al. at Zomba Central Hospital in Malawi, who found that caregivers often have limited knowledge about antibiotic use and resistance (21). This lack of knowledge leads to inappropriate practices such as self-medication, using leftover antibiotics, and buying antibiotics without a prescription. Public awareness, community-led interventions, and targeted education are crucial for educating caregivers about medication management, requiring collaboration between pharmacists and physicians (43).

The findings indicated a concerning diversity in dosing

tool selection. Syringes were the most commonly used tool, followed by medicinal spoons and household teaspoons. The preference for syringes somewhat aligns with findings from other studies where syringes were the most common device used in children aged 1 to 5 years old (44–46). However, a significant number of caregivers in our study also reported using alternative methods like medicinal spoons, household teaspoons, and household cups. This is inconsistent with recommendations that stress the use of calibrated syringes for accurate dosing, particularly for medications like antibiotics where precise measurement is crucial (47). Educational interventions should stress the importance of accurate dosing devices, especially for younger children, and caregivers should use the provided dosing tool to prevent errors (48).

A positive finding of this study was that 96.5 % of caregivers practiced good hygiene when using dosing tools. This aligns with the emphasis on handwashing and proper cleaning procedures highlighted in medication safety guidelines set by organizations like the WHO (49). Similar high rates of hygiene practices were reported in a study by Wana et al., who found that the majority of caregivers followed proper handwashing procedures before administering medication to children (50). This consistency highlights the potential effectiveness of educational interventions that emphasize hygiene practices alongside medication administration techniques (51).

The study identified positive trends in caregiver satisfaction with pharmacist counseling. Caregivers reported significantly higher satisfaction scores for both the time spent discussing medications and the adequacy of counseling, with high percentages of caregivers receiving counseling on crucial topics like side effects, administration frequency, and reconstitution techniques. The effectiveness of pharmacist counseling in promoting proper medication administration was highlighted. These results are consistent with findings from similar studies. For example, a study at Woldia Comprehensive Specialized Hospital revealed that patients who received comprehensive counseling from pharmacists exhibited significantly higher satisfaction levels (42). Furthermore, a study by Lock et al. on the effects of pharmacist-led counseling on pediatric antibiotic suspension reconstitution among rural parents highlighted that the adequacy of counseling was a significant predictor of correct antibiotic use (52). Another study by Hu et al. demonstrated significant improvements in caregivers' knowledge of antibiotic use following educational interventions, particularly emphasizing the effectiveness of one-on-one education over group sessions (38).

LIMITATIONS

This study provides valuable insights into caregiver knowledge and practices regarding antibiotic suspensions in Beirut, Lebanon. However, it is important to acknowledge some limitations. The participants were recruited through convenience sampling at pharmacies, which may have introduced selection bias. Additionally, the findings are specific to Beirut and may not be generalizable to other regions with different healthcare systems, cultural practices, or socioeconomic disparities. Furthermore, relying solely on self-reported practices through questionnaires may lead to recall bias. Participants might not accurately remember specific actions or may tend to underreport certain behaviors. Finally, the study did not comprehensively explore certain factors that could influence caregiver knowledge and practices, such as access to healthcare professionals or preferred sources of information on medication use. Despite these limitations, the study's adequate sample size and focused methodology enhance its statistical power and value for future research and intervention development.

CONCLUSION

This study conducted in Beirut, Lebanon, found that caregivers generally understood medication instructions and followed prescribed dosages. However, they often deviated from recommended practices, such as using cold

mineral water for reconstitution and not following proper techniques. Storage practices also did not meet optimal recommendations. To minimize antibiotic resistance, a comprehensive approach is recommended, including educational campaigns, strengthening pharmacist training, and incorporating visuals into medication leaflets. These interventions can promote responsible antibiotic use among caregivers, improving health outcomes and mitigating the global threat of antibiotic resistance.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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ETHICAL CONSIDERATIONS

Ethical clearance for this study was obtained from the LIU University research committee.

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آراء وممارسات مقدمي الرعاية فيما يتعلق بمعلقات المضادات الحيوية للأطفال في لبنان

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ملخص

يواجه لبنان التحدي الملح المتمثل في إساءة استخدام المضادات الحيوية للأطفال. ولمعالجة هذه المشكلة، أجرينا دراسة مقطعية في بيروت تهدف إلى تقييم معرفة وممارسات مقدمي الرعاية فيما يتعلق بمعلقات المضادات الحيوية لدى الأطفال. تمت مقابلة ما مجموعه 113 من مقدمي الرعاية في الصيدليات لتقييم فهمهم لتعليمات الدواء، ودقة الجرعات، وممارسات التخزين/التخلص، والرضا عن استشارات الصيدلي. أشارت النتائج إلى أنه في حين أظهر 67.3% من مقدمي الرعاية فهمًا واضحًا لتعليمات الدواء واتبع 63.7% إعادة التركيب السليم، فقد ظهرت ممارسات مقلقة. والجدير بالذكر أن 71.7% من مقدمي الرعاية قاموا بتخزين بقايا المعلقات في المطابخ. ومع ذلك، فإن 56.6% تخلصوا منها بشكل صحيح. إن الاستخدام الواسع النطاق للمياه المعدنية الباردة كمخفف (66.4٪) يتناقض مع المبادئ التوجيهية. لعب الصيادلة دورًا حاسمًا، حيث تلقى 69.1% من مقدمي الرعاية الاستشارة، ولكن متوسطها 3.7 دقيقة فقط، مما يشير إلى وجود مجال للتحسين. وقد ظهرت ارتباطات مهمة بين الفهم الدقيق وإعادة التكوين السليم ($p < 0.001$). وتسلط هذه النتائج الضوء على الحاجة إلى تعليمات أكثر وضوحا لمقدمي الرعاية، ومبادرات تعليمية بشأن التخزين والتخلص المناسبين، وتعزيز التدريب للصيادلة. إن معالجة هذه الجوانب يمكن أن تحسن النتائج الصحية في لبنان وتساهم في المعركة العالمية ضد مقاومة المضادات الحيوية.

الكلمات الدالة: استخدام معلق المضادات الحيوية؛ مقدمو الرعاية؛ المعرفة والممارسات؛ إدارة الأدوية؛ التحسين؛ الاستشارة الصيدلانية.

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Assessing Minipigs as Superior Non-Rodent Pre-Clinical Models: Insights from Plasma Protein Binding and Metabolism of Marketed NSAIDs Compared Across Species

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ABSTRACT

As per regulatory authorities' requirements, pre-clinical studies need to be conducted in at least one rodent and one non-rodent species. Usually, dogs are considered the non-rodent pre-clinical species of choice even though minipigs and monkeys are physiologically closer to humans than dogs. The aim of this study was to demonstrate that minipigs may be a better model for pre-clinical studies compared to dogs for some drug classes. In the present in vitro study, plasma protein binding and metabolic stability in liver microsomes of nine marketed non-steroidal anti-inflammatory drugs (NSAIDs) was evaluated in minipig, dog, monkey, and human species. Eight out of nine tested NSAIDs showed statistically similar plasma protein binding in minipig and human plasma which was different from dog and monkey plasma. Similarly, drug metabolism assays showed similar metabolism in minipig and human liver microsomes, which was different compared to dog and monkey liver microsomes. The results from both the assays showed greater similarity between minipigs and humans suggesting the use of minipig species as a better pre-clinical non-rodent model for NSAIDs instead of the conventional dog species. Additionally, the use of the more accessible minipig species may help in saving time and resources during pre-clinical studies and may help the safety studies in humans during later stage clinical trials.

Keywords: Minipig; plasma protein binding; equilibrium dialysis; NSAIDs.

INTRODUCTION

Successful pre-clinical studies during drug development are a steppingstone in a drug's success story. The selection of pre-clinical species is a critical factor in a new drug development program as it affects the safety in humans and the design of further clinical studies. As per regulatory requirement, a new chemical entity (NCE) must be preclinically tested in at least one rodent and one non-rodent species before conducting clinical studies in humans to ensure safety in humans. For pre-clinical studies, dogs and monkeys (non-human primates) are traditionally used as non-rodent species. Dogs are accessible, easy to handle, and extensive prior toxicological safety assessment data exists in literature. Monkeys are less accessible but are closest to humans in terms of genetic similarity. However, use of dogs and monkeys is restricted by societal constraints, certain physiological differences, as well as ethical issues. Minipigs are the other alternate non-rodent species for pre-clinical studies.

They have many anatomical, physiological and biochemical similarities with humans relative to other non-rodent species [1, 2-13]. Since then, use of minipigs have been increased in biomedical research. In the European Union (EU), currently more than 60,000 pigs are used per

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year for scientific procedures [7].

Gottingen minipigs are the most widely used. In the early 1960s, Göttingen University first developed the Göttingen strain [7]. The benefit of the Göttingen strain is that it is a white non-pigmented and small-size minipig (adult average body weight of 30–40 kg). It is of very high parasitic and microbiological quality [6-7].

The cardiovascular, digestive, and urogenital systems of the minipig are highly comparable to the human systems [1-9]. The anatomy of the skin is relatively close to human skin and hence it can also be used for dermal studies. Metabolic activity and enzymatic processes in minipigs have close parallels to the humans. The minipigs are sensitive to a wide variety of chemicals and drugs which have been advantageous over the traditional non-rodent species in relation to specific responses to particular drug classes. Minipigs can be used for all routes of administration as parenterally or orally, and in many cases for metabolic or pharmacological reasons minipigs are preferable to dogs or non-human primates [5-6].

Moreover, pharmacokinetics of drug candidates in humans has shown more resemblance to minipigs than to dogs. This result is primarily due to the species differences observed in metabolizing enzymes and drug transporters. Cytochrome P450 pattern of enzymes in minipigs has been reported and has shown similarities to humans [5]. Similarly, expression of P-gp and CYP3A in livers and small intestines of foetal, neonatal, juvenile, and adult Göttingen minipigs has been assessed by immunohistochemical methods and shown to be comparable to humans [5].

There are important differences between minipigs and dogs which in some cases favour the use of minipigs instead of the conventionally used dogs as a non-rodent model in safety evaluation [6]. Minipigs are the recommended non-rodent species for drug candidates that are metabolized by aldehyde oxidase (AO), N-acetyltransferases (NAT1 and NAT2) or cytochrome P450 (CYP2C9) enzymes which are not expressed in dogs [13]. Another advantage of using minipigs is the lack of emesis which is a major drawback in dogs for certain drugs like anticancer and NSAIDs. Emesis in dogs during pre-clinical studies can generate incorrect or unreliable data which may later affect the clinical studies in humans [37].

Even though minipig species has great potential based on the genetic background data, it is still necessary to corroborate these findings using experimentation. The current manuscript presents an in vitro comparison of plasma protein binding and metabolism in dog, minipig, monkey and human liver microsomes for different NSAIDs. Although the data of plasma protein binding and metabolism of these NSAIDs are available in dogs and humans, the data from minipigs and monkeys are not available. Based on the results, three non-rodent species dogs, minipig and monkey are evaluated to select the better non-rodent model for preclinical studies.

METHODS

Reagents used.

NSAIDs in active pharmaceutical ingredient (API) form of celecoxib, diclofenac, ibuprofen, ketoprofen, flurbiprofen, indomethacin, meloxicam, piroxicam and naproxen were purchased from Sigma-Aldrich (St. Louis, Missouri). All other chemicals were of reagent grade and purchased from commercial sources. Dog plasma (Beagle, male) and minipig plasma (Gottingen, male) were purchased from Palamur Biosciences (Hyderabad, India). Monkey plasma (Cynomolgus, male) was procured from BioIVT, NY, USA and human plasma (Caucasian, male) was obtained from Bio Ally (Bangalore, India). Plasma used in the assays was a pool of three donors and was collected in K_2EDTA anticoagulant. Dog, minipig, human and monkey liver microsomes were purchased from Sekisui Xenotech LLC (Kansas, USA).

Plasma Protein Binding by Equilibrium Dialysis Method

The 96-well equilibrium dialysis apparatus (HTD 96B, Gales Ferry, Connecticut, USA) and cellulose membranes

(12-14 KDa molecular weight cutoff) were purchased from HT Dialysis (Gales Ferry, Connecticut, USA). The cellulose membranes were regenerated by soaking in Milli-Q water for 15 min, followed by 15 min in 25% methanol and finally 15 min in 100 mM sodium phosphate buffer (pH 7.4). The equilibrium dialysis apparatus was assembled by placing the regenerated membranes between rows of wells of Teflon bars. The apparatus was tightly clamped to prevent any leakage during dialysis [19-22].

The pH of blank plasma from all species was adjusted to 7.4 using 0.1 N HCl and preincubated for 15 min at 37 °C. DMSO stock solutions of the compounds were spiked in the blank plasma in 1.5 mL Eppendorf microfuge tubes (0.4% final DMSO concentration) to get 10 µM final concentration. These tubes were mixed by inversion 5-6 times. Aliquots (120 µL per half-cell) of plasma spiked with drug were loaded into the donor chamber of the dialysis plate and remaining plasma in tubes were incubated at 37°C for 6 h with 5% carbon dioxide atmosphere. In receiver chamber, 120 µL of blank sodium phosphate buffer (100 mM, pH 7.4) was added. The Dialyser was sealed with adhesive sealer (Axyseal, Axygen, USA) and dialysis was conducted for 6 h at 37 °C, 60 rpm and 5% CO_2 atmosphere. After incubation for 6 h, aliquots from the donor and receiver chambers were pipetted and collected in acetonitrile containing internal standard. Similarly, aliquots from plasma tubes incubated for 6 h were collected in acetonitrile containing internal standard. LC-MS/MS was used to analyse these samples.

Binding of warfarin in human plasma (literature value approx. 1% unbound) [19,25] in one well of each row was tested as positive control to ensure membrane integrity, adequate clamp pressure and no leakage in each row of the dialysis plate.

Acceptance criteria for plasma protein binding assay: To accept and consider the compound as stable in plasma, 100 ± 30% criteria were considered at 6 h compared to 0 h samples concentration. To rule out any non-specific binding of the compound to the membrane or apparatus the percent recovery had to be within 70-130% [19].

percent unbound are reported for the compounds where percent CV (coefficient of variation) was <30% and at least 4 replicates out of 6 replicates were acceptable.

The positive control warfarin in human plasma run along with the compounds showed percent unbound of approximately 1% [19,25].

Procedure for Drug Metabolism Assay

Liver Microsomes Assay: In a 96 deep well plate 50 mM sodium phosphate buffer (pH 7.4), liver microsomes (final concentration 0.5 mg/mL), and nicotinamide adenine dinucleotide phosphate (NADPH, final concentration 1 mM) were added. The plate was kept in an incubator shaker for preincubation at 37°C, 60 rpm for 10 min. After 10 min, the compound stock solutions prepared in DMSO was spiked in the reaction mixture to obtain final compound concentration of 0.5 µM. After spiking of the compound, reaction mixture was mixed well and aliquots were taken at the time points of 0, 5, 10, 20 and 30 min and immediately quenched with acetonitrile containing internal standard rolipram/flufenamic acid.

Similarly, control experiment without NADPH was performed parallelly to investigate non-CYP metabolism. Samples were collected at 0 and 30 min and immediately quenched with acetonitrile containing internal standard rolipram/flufenamic acid.

Verapamil (highly metabolised in dog, minipig, monkey and human liver microsomes) was used as positive control along with the compounds to ensure that the liver microsomes used in the assay were metabolically active. Acceptance criteria for metabolic stability assay was that the percent remaining for positive control verapamil must be ≤ 25% at 30 min in dog, minipig, monkey and human liver microsomes.

Bioanalysis

Bioanalysis of metabolic stability and plasma protein binding samples was conducted on LC-MS/MS (Applied Biosystems API 4000 triple quadrupole, AB Sciex®, Ontario, Canada) using a Thermo C18, 4.6x50 mm, 5 µm

column. Acetonitrile with 0.1% formic acid (mobile phase B):5 mM ammonium formate with 0.1% formic acid (mobile phase A) at 70:30 ratio was used for analysis using positive mode (MH+) ionisation for diclofenac, ibuprofen, ketoprofen, meloxicam and piroxicam. Rolipram was used as internal standard for diclofenac, ibuprofen, ketoprofen, meloxicam and piroxicam analysis.

Acetonitrile (mobile phase B):5 mM ammonium acetate (mobile phase A): 70:30 was used for compounds analysis using negative mode (MH^-) for celecoxib, flurbiprofen, indomethacin and naproxen. Flufenamic acid was used as internal standard for celecoxib, flurbiprofen, indomethacin and naproxen analysis.

Each analytical run had twelve calibration curve standards, and two sets of quality control (QC) samples run at three different concentrations, low, medium, and high-quality control (LQC, MQC and HQC). LQC was selected as five times the lower limit of quantification (LLOQ), HQC was 75% of the upper limit of quantification and MQC was one intermediate quality control selected from the calibration curve standards. Batch was accepted when at least 67% of overall calibration curve met ± 20% accuracy criteria and 50% of QCs at each concentration level of QC met ± 20% accuracy criteria.

Data Analysis for Plasma Protein Binding Samples

Percent unbound of the compounds was determined using the concentrations in buffer samples (receiver compartment) and in plasma samples (donor compartment) collected from the HT Dialysis® apparatus after 6 h of dialysis using equation no 1 below.

$$\%Unbound = \left\{\frac{[Compound]_{receiver}}{[Compound]_{donor}}\right\} \times 100 \qquad (1)$$

$$\%Bound = 100 - \%Unbound$$

Percent recovery was calculated to evaluate non-specific binding by adding the concentrations in the receiver compartment and donor compartment samples and comparing that with the 6 h stability samples using equation no 2 below.

$$\%Recovery = \left\{\frac{[Compound]_{receiver} + [Compound]_{donor}}{[Compound]_{6\,h}}\right\} \times 100 \qquad (2)$$

Data Analysis of Metabolic Stability Samples: Percent remaining of parent compound at different time points was calculated using area ratio at that time point compared to zero min area ratio. The data was fitted to a one phase exponential decay equation ($A = A_0e^{-kt}$) using GraphPad Prism® software. The half-life ($t_{1/2}$) generated by the software are reported. Intrinsic clearance CL_{int} was calculated using the below equation no. 3, where k = decay rate constant (min^{-1}).

$$CL_{\text{int}} = \frac{k \times \text{volume of reaction mixture } (\mu L)}{\text{protein content (mg)}} \qquad (3)$$

Statistical Analysis: A two tailed t-test was conducted to check the percent unbound value observed was significant (S) or non-significant (NS). GraphPad Prism® software was used to calculate the p-value. The means of percent unbound considered significantly different when the p value was <0.05. p-value >0.05 was considered as non-significant difference.

RESULTS

Plasma Protein Binding: Total nine NSAIDs (celecoxib, diclofenac, ibuprofen, flurbiprofen, ketoprofen, meloxicam, piroxicam, indomethacin and naproxen) were tested for in vitro plasma protein binding in dog, minipig, monkey and human plasma to compare the binding, to find the similarity and to select the best non-rodent preclinical model. Eight out of nine NSAIDs showed similar binding in minipig and human plasma.

All nine compounds were stable up to 6 h at 37°C in dog, minipig, monkey and human plasma so the percent unbound observed is acceptable and reliable. The literature values available for human plasma binding for these reported NSAIDs are consistent with the observed values in this study [19,25]. The average percent remaining at 6 h was >95 % for all the compounds.

Percent recovery was 70-130% indicating that there was no non-specific binding of the compounds to the cellulose membranes or any leakage in HT Dialysis® apparatus during dialysis period of 6 h.

The percent unbound, percent stability and percent recovery in dog, minipig, monkey and human with standard deviation (SD) are reported in Table 1 and Table 2.

Table 1. Percent Unbound of the NSAIDs with SD in Dog, Minipig, Monkey and Human Plasma

% Unbound				
Compound (NSAIDs) Name	**Dog**	**Minipig**	**Human**	**Monkey**
Celecoxib	2.17 ± 1.06	1.28 ± 0.65	1.50 ± 0.22	2.42 ± 0.72
Diclofenac	1.96 ± 1.27	1.06 ± 0.57	0.86 ± 0.90	0.99 ± 0.40
Ibuprofen	0.41 ± 0.15	0.13 ± 0.06	0.56 ± 0.34	0.43 ± 0.31
Flurbiprofen	3.60 ± 1.68	0.54 ± 0.54	0.36 ± 0.08	0.51 ± 0.28
Ketoprofen	5.91 ± 1.93	1.30 ± 0.96	2.47 ± 0.80	7.06 ± 0.72
Meloxicam	2.44 ± 0.38	0.68 ± 0.55	0.25 ± 0.10	0.69 ± 0.28
Piroxicam	5.16 ± 0.69	1.29 ± 0.52	0.43 ± 0.01	0.74 ± 0.16
Indomethacin	1.84 ± 0.74	1.22 ± 0.18	0.69 ± 0.16	0.62 ± 0.55
Naproxen	2.07 ± 1.36	0.5 ± 0.43	0.68 ± 0.34	0.48 ± 0.49
Warfarin (Positive Control)*	-	-	1.45 ± 0.40	-
* Warfarin binding (approx. 1% unbound as per literature) in human plasma at 10 µM was run as positive control to check the validity of dialysis apparatus and membrane integrity.				

Table 2. Percent Stability and Percent Recovery of the NSAIDs in Dog, Minipig, Monkey and Human Plasma at 6 h, 37°C

% Stability and % Recovery at 6 h, 37 °C								
Species	**Dog**		**Minipig**		**Human**		**Monkey**	
Compound (NSAIDs) Name	**%Stability**	**%Recovery**	**%Stability**	**%Recovery**	**%Stability**	**%Recovery**	**%Stability**	**%Recovery**
Celecoxib	95	103	98	102	96	98	113	86
Diclofenac	96	107	99	105	95	104	93	106
Ibuprofen	94	116	96	106	95	101	93	100
Flurbiprofen	93	93	103	95	96	106	96	101
Ketoprofen	111	98	118	100	118	94	109	98
Meloxicam	105	97	100	110	101	99	85	127
Piroxicam	101	86	100	98	96	98	75	125
Indomethacin	93	129	93	128	84	94	75	105
Naproxen	109	94	101	103	97	98	127	88
Warfarin	-	-	-	-	93	101	-	-
Considering experimental errors, 70-130% stability and 70-130%recovery is acceptable.								

To check the percent unbound values are similar or significantly different between the evaluated four species, a two tailed t-test was conducted comparing dog with human, minipig with human and monkey with human. The p-values were calculated using GraphPad Prism® software.

The statical analysis data for comparison of plasma protein binding in different species are presented in Table 3.

Table 3. Statistical Analysis of Percent Unbound Values and Summary of p-values Comparing Dog, Minipig and Monkey to Human Species

* p-Value by two tailed t-test			
Compound (NSAID) name	**Dog vs Human**	**Minipig vs Human**	**Monkey vs Human**
Celecoxib	0.017 (S)	0.5542 (NS)	0.1318 (NS)
Diclofenac	0.036 (S)	0.1064 (NS)	0.2036 (NS)
Ibuprofen	0.2716 (NS)	0.0743 (NS)	0.6754 (NS)
Flurbiprofen	0.0189 (S)	0.4028 (NS)	0.3833 (NS)
Ketoprofen	0.0231 (S)	0.1208 (NS)	<0.0001 (S)
Meloxicam	<000.1 (S)	0.2085 (NS)	0.0051 (S)
Piroxicam	<000.1 (S)	0.009 (S)	0.0149 (S)
Indomethacin	0.0417 (S)	0.0771 (NS)	0.5261 (NS)
Naproxen	0.0469 (S)	0.6416 (NS)	0.3288 (NS)
* p-Values were calculated by comparing the six replicates of percent unbound values between human and dog, human and minipig, human and monkey. * p-value <0.05 means significantly different (S) while >0.05 means not significantly different (NS).			

Metabolic Stability in Liver Microsomes: In vitro metabolic stability assay results of these NSAIDs in dog, minipig, monkey and human liver microsomes are presented below:

After 30 min of incubation in pH 7.4 buffer and at 37 °C, it was observed that diclofenac and ibuprofen were metabolized by minipig and human liver microsomes however was stable in dog and monkey liver microsomes at 37 °C.

Intrinsic clearance values of piroxicam were similar in minipig and human liver microsomes and piroxicam was stable in monkey and human liver microsomes with low intrinsic clearance.

Indomethacin was unstable in minipig liver microsomes and stable in dog, monkey and human liver microsomes, Meloxicam was moderately stable in dog and minipig but was stable in monkey and human liver microsomes.

The positive control verapamil that run along with the NSAIDs was unstable (half-life was 2.3-6.5 min) in dog, minipig, monkey and human liver microsomes with high intrinsic clearance values (>200 µL/min/mg protein) indicating that the liver microsomes used in this study were metabolically active [36].

The percent metabolism, half-life ($t_{1/2}$) and CL_{int} values of the NSAIDs in dog, minipig, monkey and human liver microsomes are summarized in Table 4 and Table 5.

Table 4. Percent Metabolism of the NSAIDs at 30 min in Dog, Minipig, Monkey and Human Liver Microsomes

% Metabolism at 30 min				
Compound (NSAIDs) Name	**Dog**	**Minipig**	**Human**	**Monkey**
Celecoxib	56	74	38	42
Diclofenac	0	28	88	0
Ibuprofen	0	62	18	16
Ketoprofen	0	0	0	0
Flurbiprofen	14	100	64	0
Meloxicam	23	48	0	0
Piroxicam	0	8	1	0
Indomethacin	0	25	0	11
Naproxen	0	21	0	6
Verapamil (Positive Control)	89	99	96	97

Table 5. Half-life ($t_{1/2}$) and CL_{int} Values of the NSAIDs in Dog, Minipig, Monkey and Human Liver Microsomes

Half life ($t_{1/2}$) and CL_{int} Values								
Compound (NSAIDs) Name	**Dog**		**Minipig**		**Human**		**Monkey**	
	Half Life (min)	**CL_{int} (µL/min/mg protein)**	**Half Life (min)**	**CL_{int} (µL/min/mg protein)**	**Half Life (min)**	**CL_{int} (µL/min/mg protein)**	**Half Life (min)**	**CL_{int} (µL/min/mg protein)**
Celecoxib	24	61	15	92	>30	33	26	54
Diclofenac	>30	7	>30	22	10	145	0	0
Ibuprofen	>30	2	23	59	>30	21	>30	12
Ketoprofen	>30	0	>30	0	>30	0	>30	0
Flurbiprofen	>30	7	9	155	23	60	0	0
Meloxicam	>30	19	>30	44	>30	0	12	0
Piroxicam	>30	0	>30	9	>30	9	>30	0
Indomethacin	>30	0	>30	21	>30	0	>30	9
Naproxen	>30	0	>30	18	>30	0	>30	2
Verapamil (Positive Control)	6.5	214	2.3	611	6	226	3.1	446

DISCUSSION

The present study was conducted using nine NSAIDs to evaluate if minipig was a better model to predict human safety and efficacy relative to the conventional dog species. There are drawbacks in using dog species in preclinical studies such as its dissimilarities with human, potential for emesis, and certain social constraints. [1, 37] The current investigation is based on the similarity in anatomy and physiology between minipig and human species. Two in vitro ADME characteristics, plasma protein binding and metabolic stability in liver microsomes were compared in dog, minipig, monkey and human species and it was shown that minipigs are a good alternate during drug development.

Plasma protein binding is an important parameter to comprehend the efficacy of a drug in different pre-clinical species. The fraction of unbound drug in conjunction with other in vitro ADME parameters is used to understand in vivo properties of the compound including its availability at the target of interest. The fraction unbound is also used to calculate the dose to be administered. Binding of the drugs to the proteins of different animals and humans may not be similar hence it can affect the efficacy and safety of a compound [19-24].

If the drug is highly and tightly bound (slow dissociation) then it may result in restricted distribution of drug into target tissue (reduced Vd), decreased metabolism, clearance and prolonged half-life, retention of drug in plasma compartment resulting in limited brain penetration, and higher requirement of loading doses but lower maintenance doses [22].

The current in vitro plasma protein binding assay for nine reported NSAIDs was conducted in dog, minipig, monkey and human plasma by equilibrium dialysis method using a HT Dialysis® apparatus [21] to compare the binding in three non-rodent model (dog, minipig and monkey) with human. pH of the plasma was adjusted to 7.4 before the study because the plasma protein binding is pH dependent [23-24]. To keep the plasma pH constant, CO_2 incubator was used, and 5% CO_2 was supplied throughout the incubation time of 6 h.

For evaluation of plasma protein binding by the equilibrium dialysis method, the compound must be stable for 6 h and soluble in plasma. If a compound has poor solubility, instability in plasma, and/or high nonspecific binding, then alternate methods such as ultracentrifugation and ultrafiltration need to be employed. However, in this study all the 9 compounds (NSAIDs) along with the

positive control warfarin were soluble and stable up to 6 h in dog, minipig, monkey and human plasma. The percent stability of the NSAIDs were in the range of 75-127%. The percent recovery for the NSAIDs were 86-129% hence there were no non-specific binding of the tested NSAIDs to the dialysis membranes or HT Dialysis® apparatus.

The plasma protein binding results showed correspondence between minipig and human statistically. Eight out of nine evaluated NSAIDs showed significantly similar binding in minipig and human, vs. only one case for dog and human. In many cases, monkey was also closer to human than dog (six compounds out of nine compounds showed similarity). However, there are more constraints and challenges in conducting monkey studies [13] therefore minipig may be a better non-rodent model for pre-clinical studies for NSAIDs.

Similarly, the four species, dog, minipig, monkey and human, were compared in metabolic stability study in liver microsomes. The positive control verapamil that run along with the NSAIDs was unstable (half-life was 2.3-6.5 min, CL_{int} >200 µL/min/mg protein) in dog, minipig, monkey and human liver microsomes indicating that the liver microsomes used in this study were metabolically active [32,36].

Diclofenac and ibuprofen were metabolized more by minipig and human liver microsomes but were stable in dog and monkey liver microsomes at 30 min. This result can be explained by understanding the expression of various CYP isozymes in the different species. Diclofenac and ibuprofen are reported substrates for cytochrome P450 (CYP) 2C9 [36,37], CYP 2C9 like CYPs are not expressed in dog but expressed in minipig and human liver microsomes hence metabolism of the diclofenac and ibuprofen was observed in minipig and human but not in dog.

Piroxicam showed similar intrinsic clearance in minipig and human liver microsomes and no intrinsic clearance in monkey and human. Indomethacin was stable in dog, monkey and human but was unstable in minipig liver microsomes. Flurbiprofen was more metabolized by minipig and human liver microsomes compared to dog and monkey liver microsomes. Meloxicam was metabolized in dog and minipig but was stable in monkey and human liver microsomes.

To conclude, the drugs which are metabolized by CYP 2C9, CYP 2C9 like CYPs, aldehyde oxidase (AO), NAT and OAT3 showed similar metabolism in minipig and human. If a drug candidate is known to be metabolized by these enzymes-minipig may be the best choice for preclinical studies.

CYP content in liver microsomes (nM/mg of protein) is also an important factor to be considered in drug metabolism. This CYP content could be the reason for high metabolism of indomethacin and meloxicam in minipig compared to human. CYP content present in liver microsomes differs in the different species [39], human CYP content - 0.307±0.16 nM/mg of protein (n=18), minipig CYP content - 0.81±0.15 (n=9), monkey CYP content 1.03±0.11 (n=5), and dog CYP content 0.39±0.04 (n=6) [38,39].

The recent research in oligonucleotides showed the minipig is a suitable non-rodent model in the safety assessment of single stranded oligonucleotides [41-43] and immunosafety sciences [44-45]. Pharmaceutical industries started using minipigs as pre-clinical non-rodent model recently [46].

The current study showed that the plasma protein binding and hepatic metabolism was similar between minipig, and human compared to dog and monkey in case of NSAIDs. However, the results of the current study are limited to NSAIDs and the plasma protein binding and hepatic metabolism for other drug classes may show differences between minipig and human species. Hence different classes of drugs should be investigated and compared across species to determine the best or most suitable pre-clinical non-rodent species for pre-clinical trials.

CONCLUSION

The objective of this in vitro study was to compare the plasma protein binding and metabolism characteristics of nine NSAIDs in different non-rodent preclinical species to find the relatively closest species to human. The results of

the current investigation corroborated the anatomical and physiological similarities of minipig with human and showed that the minipig results were comparable to human than dog and monkey for both the ADME assays with these NSAIDs. Hence minipig may prove to be a better preclinical non-rodent model to predict human safety and efficacy for studying NSAIDs drug class. The same ADME studies can be extended to other drug classes to understand if such a correspondence exists in those cases. Before conducting in vivo toxicological studies for new chemical entities (NCEs), these ADME assays might be helpful for selecting the best pre-clinical model.

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تقييم الخنازير المصغرة كنماذج ما قبل السريرية المتفوقة غير الإكلينيكية: رؤى من ارتباط بروتين البلازما والتمثيل الغذائي لمضادات الالتهاب غير الستيروئيدية المسوقة مقارنة بين الأنواع

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ملخص

وفقًا لمتطلبات السلطات التنظيمية، يجب إجراء دراسات ما قبل السريرية على الأقل على نوع واحد من القوارض ونوع واحد غير القوارض. وعادةً ما تُعتبر الكلاب من الأنواع غير القوارض المفضلة للدراسات ما قبل السريرية على الرغم من أن الخنازير الصغيرة والقرود أقرب فسيولوجيًا إلى البشر من الكلاب. وكان الهدف من هذه الدراسة هو إثبات أن الخنازير الصغيرة قد تكون نموذجًا أفضل للدراسات ما قبل السريرية مقارنة بالكلاب لبعض فئات الأدوية. في الدراسة الحالية في المختبر، تم تقييم ارتباط بروتين البلازما والاستقرار الأيضي في ميكروسومات الكبد لتسعة أدوية مضادة للالتهابات غير الستيرويدية (NSAIDs) مسوقة في أنواع الخنازير الصغيرة والكلاب والقرود والبشر. أظهرت ثمانية من أصل تسعة مضادات التهاب غير ستيرويدية تم اختبارها ارتباطًا إحصائيًا مماثلًا لبروتين البلازما في بلازما الخنازير الصغيرة والبشر والذي كان مختلفًا عن بلازما الكلاب والقرود. وبالمثل، أظهرت اختبارات استقلاب الدواء استقلابًا مشابهًا في ميكروسومات الكبد لدى الخنازير الصغيرة والبشر، والذي كان مختلفًا مقارنة بميكروسومات الكبد لدى الكلاب والقرود. أظهرت نتائج كلا الاختبارين تشابهًا أكبر بين الخنازير الصغيرة والبشر، مما يشير إلى استخدام أنواع الخنازير الصغيرة كنموذج غير قوارض أفضل قبل السريرية لمضادات الالتهاب غير الستيرويدية بدلاً من أنواع الكلاب التقليدية. بالإضافة إلى ذلك، قد يساعد استخدام أنواع الخنازير الصغيرة الأكثر سهولة في توفير الوقت والموارد أثناء الدراسات قبل السريرية وقد يساعد في دراسات السلامة لدى البشر أثناء التجارب السريرية في المراحل اللاحقة.

الكلمات الدالة: الخنازير الصغيرة، ربط بروتين البلازما، غسيل الكلى المتوازن، مضادات الالتهاب غير الستيرويدية.

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Exploring Payment Refusals for Health Insurance-Covered Medical Costs: A Hospital Case Study in Vietnam

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ABSTRACT

Adjudication and rejection of payment for healthcare costs covered by health insurance pose challenges in maintaining healthcare operations and ensuring the rights of patients and healthcare staff at hospitals. Research on this issue is limited globally and in Vietnam. This study was conducted to understand the current status of refusal to pay medical examinations and treatment costs covered by health insurance at a hospital in Can Tho, Vietnam, and to explore the reasons for payment refusal. A cross-sectional descriptive study retrieved data on health insurance-covered medical expenses that were refused at a hospital in Can Tho, Vietnam, over three years from January 1, 2020, to December 31, 2022. This study found that from 2020 to 2022, the number of settlements was consistently lower than the proposed costs. The highest refused payment amount in 2022 was 334,232,049 Vietnamese dong (0.4%). Inpatient health insurance-covered medical expenses faced greater payment refusal than outpatient expenses. Medications, surgical procedures, and bed charges were the top three reasons for payment refusal from 2020 to 2022. The primary cause of payment refusal remains predominantly associated with medication costs. This poses challenges in cost management and necessitates measures to address the issue of payment refusal within the health insurance system.

Keywords: health insurance; drug cost; healthcare provider; hospital; Vietnam.

INTRODUCTION

Health insurance (HI) is a form of insurance applied in the field of healthcare and is one of the 9 contents of social insurance stipulated in the International Labor Organization (ILO) Convention No. 102 of June 28, 1952, on minimum standards for various social insurance benefits [1]. In Vietnam, HI is considered a mandatory form of insurance applied to specific subjects, as regulated by the 2008 Health Insurance Law to provide healthcare without profit motives, organized and implemented by the Vietnamese State [2]. HI is a significant social policy, a special type of insurance with profound humanitarian meaning, and a long-term strategy in community healthcare and health improvement, contributing significantly to achieving social justice in

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healthcare and protecting people's health [2,3]. As housing disparities drive health inequality, with high costs and poor conditions worsening risks for vulnerable groups, HI plays a crucial role in ensuring equitable access to healthcare [4]. To ensure equity in healthcare and benefits from HI for the entire population of Vietnam, the government has issued numerous policies over the years to expand the coverage of the HI participation rate, resulting in an increase rate from 90.0% (2019) to 92.04% (2022) [5–8]. However, ensuring high-quality services for HI participants remains a challenge. A previous study showed that patients with military insurance reported significantly higher satisfaction with pharmaceutical services, while no difference was observed among government, private, and uninsured groups [9]. This highlights the need to improve service delivery for equitable healthcare benefits.

In Vietnam, the sources contributing to HI and other legal revenue sources are collectively referred to as HI funds. The majority of this fund (90%) is used to cover the costs of medical examinations and treatments for HI participants, with a specific percentage allocated for the establishment of a reserve fund for HI medical examinations and management fees [2,10]. The HI medical examination and treatment fund are utilized for activities such as inpatient and outpatient care, functional recovery, regular pregnancy check-ups, childbirth, emergency patient transportation or transfer for continuous treatment, and payments for drugs, chemicals, medical supplies, and technical services according to the Ministry of Health's prescribed list. The payment of HI medical examination and treatment costs is carried out by the HI fund management organization through legal procedures regulated by the Vietnamese State [2,10]. To avoid misappropriation or misuse of HI funds, the Vietnamese Health Insurance Law stipulates the rejection of payment for medical examination and treatment costs that do not comply with the law or the terms of the health insurance contract [11].

The reasons for HI payment refusal include errors by healthcare providers in adhering to legal regulations related to healthcare activities and HI payment procedures. The rejected costs often involve medication, medical supplies, and services beyond the prescribed list or not meeting the Ministry of Health's payment conditions. Administrative procedure errors related to patient administrative information and payment procedures also contribute to payment refusals. Furthermore, the professional factors of healthcare providers can lead to payment rejection [12].

The refusal of health insurance payments for medical examination and treatment costs is a topic of significant concern in healthcare facilities [12–14]. Presently, healthcare facilities in Vietnam face challenges in HI payment for medical examination and treatment costs, while social insurance agencies tighten the scrutiny and settlement of HI medical examination and treatment costs. Many hospitals reject HI payments, with amounts reaching tens of billion VND. Specifically, the amounts refused by HI nations were 23 billion VND (2014), 258 billion VND (2016), and 480 billion VND (2017) [15]. A 2016 study conducted at the District General Hospital of Hoai Duc, Hanoi, reported that the hospital had 516,607,886 VNDs rejected by social insurance, with laboratory tests, drugs, and diagnostic imaging fees being the top three expense categories, accounting for 51.04%, 20.12%, and 14.22% of the total refused costs, respectively [14]. The reasons for rejection were attributed to a lack of understanding of the payment mechanism. In 2017, the 7th Military Hospital had 975 million VNDs refused by social insurance, accounting for 1.28% of the total proposed payment. The top three expense categories with the highest rejection rates were medication, laboratory tests, and surgical procedures, accounting for 49.96%, 18.49%, and 15.24% of the total refused costs, respectively [13]. In 2018, the Central General Hospital of An Giang had 1,154,290,399 VNDs refused by social insurance due to improper prescription of drugs, inappropriate ordering of technical services, discrepancies in the cost of color phototyping for cardiac ultrasound, incorrect patient transfer, and incorrect bed payment [16].

Despite the significance of health insurance for the overall healthcare system and the health of the population, research on health insurance payment refusals is limited globally and in Vietnam. This study aimed to understand the current status of refusal to pay medical examinations and treatment costs covered by health insurance at a hospital in Can Tho, Vietnam, and to explore the reasons for payment refusal.

METHOD

This cross-sectional descriptive study retrospectively analyzed data on health insurance-covered medical expenses that were refused payment at a hospital in Can Tho, Vietnam, over a three-year period from January 1, 2020, to December 31, 2022. Can Tho, a centrally governed city, serves as the hub of the Mekong Delta region, encompassing a natural area of 1,401.61 km^2 [17]. The healthcare system in Can Tho comprises 27 hospitals, 4 health centers, 80 medical stations, and over 1,208 private healthcare facilities. In 2020, the physician-to-population ratio was 16.83, and the pharmacist-to-population ratio was 4.32, both of which exceeded the national averages [18].

This research focuses on data related to health insurance payment refusals for medical expenses at a specific hospital in Can Tho, Vietnam. A whole-population sampling method was employed, and data were collected through forms from the hospital's healthcare management software. The dataset includes all relevant information regarding health insurance activities at the hospital, such as payment settlement records, health insurance-covered medical expense invoices, cost summary sheets, outpatient and inpatient health insurance examination results, and payment reports managed by the hospital's Planning Department.

Continuous quantitative data on the health insurance settlement for medical expenses at the hospital (VND) were collected, including health insurance funds, total health insurance-covered expenses, proposed health insurance medical expenses, settled health insurance medical expenses, health insurance medical expenses refused, and refusals directly through examination. Continuous quantitative data on health insurance-covered medical examination and treatment costs (VNDs), including bed-day expenses, medication expenses, surgical procedure expenses, laboratory test expenses, medical examination expenses, medical supplies expenses, imaging and diagnostic expenses, and blood expenses, were collected. Continuous quantitative data on the reasons for the rejection of health insurance medical expenses (VND) were collected, including diagnostic imaging - functional exploration, surgery – operation, medication, bed fees, examination fees, medical supplies, and laboratory tests.

Data synthesis and processing were conducted using Microsoft Excel software. Descriptive statistics, including frequency and percentage, were employed to summarize the study's dataset. This study analyzed a total of 100,733 health insurance-covered medical expense records submitted by the hospital in 2020, 66,521 records in 2021, and 90,479 records in 2022.

RESULTS

In the period 2020-2022, the proposed health insurance-covered medical expenses reached their highest value in 2022, accounting for 81.9% of the total health insurance medical expenses (Table 1). While this expense was higher than the allocated budget for health insurance-covered medical expenses in 2020, it was lower than those in 2021 and 2022. Through the assessment process, the settled amounts consistently remained lower than the proposed expenses over the years. The highest refused payment occurred in 2022, amounting to 334,232,049 VND, with the entire sum stemming from direct refusals during the assessment process.

Table 1. Results of Health Insurance Settlement for Medical Expenses at the Hospital during the Period 2020-2022

No.	Index	Value (VND)		
		2020	2021	2022
1	Health Insurance Fund	71,540,575,225	71,321,839,821	80,628,676,451
2	Total Health Insurance-Covered Expenses	89,576,871,220	79,669,335,634	92,967,558,420
3	Proposed Health Insurance Medical Expenses	71,938,945,210	66,453,740,777	76,186,025,874
4	Settled Health Insurance Medical Expenses	71,697,255,504	66,244,103,208	75,851,793,825
5	Health Insurance Medical Expenses Refused	241,689,706	209,637,569	334,232,049
5.1	Refused directly through examination	241,689,706	209,637,569	334,232,049

Over the years from 2020 to 2022, the value of health insurance medical expenses for inpatient care has consistently exceeded that of outpatient care (Table 2). The total outpatient medical expenses fluctuated, from 18.814 billion VND in 2020 to 13.471 billion VND in 2021 and increasing to 18.453 billion VND in 2022. Total inpatient medical expenses also fluctuated, from 70.762 billion VND in 2020 to 66.198 billion VND in 2021 and increasing to 74.515 billion VND in 2022. For outpatient care, medication expenses contributed the most, while medical supply expenses accounted for the highest proportion of inpatient health insurance medical expenses.

Table 2. Structure of health insurance-covered medical examination and treatment costs

STT	Expense Categories	Outpatient		Inpatient	
		Value (VND)	Percentage (%)	Value (VND)	Percentage (%)
2020					
1	Bed-day Expenses	0	0.00	9,656,373,100	13.65
2	Medication Expenses	11,077,248,028	58.88	13,738,341,092	19.41
3	Surgical Procedures Expenses	455,443,600	2.42	17,187,152,440	24.29
4	Laboratory Test Expenses	1,587,214,000	8.44	5,320,057,100	7.52
5	Medical Examination Expenses	3,174,696,900	16.87	66,556,150	0.09
6	Medical Supplies Expenses	33,468,562	0.18	19,457,541,048	27.50
7	Imaging and Diagnostic Expenses	2,480,473,700	13.18	4,073,325,500	5.76
8	Blood Expenses	5,952,000	0.03	1,263,028,000	1.78
Total		**18,814,496,790**	**100**	**70,762,374,429**	**100**
2021					
1	Bed-day Expenses	0	0.00	9,076,654,550	13.71
2	Medication Expenses	8,221,788,708	61.03	15,670,536,891	23.67
3	Surgical Procedures Expenses	280,774,200	2.08	13,905,703,782	21.01
4	Laboratory Test Expenses	1,133,030,900	8.41	5,394,864,100	8.15
5	Medical Examination Expenses	2,000,316,900	14.85	49,021,600	0.07
6	Medical Supplies Expenses	7,334,765	0.05	16,160,533,638	24.41

STT	Expense Categories	Outpatient		Inpatient	
		Value (VND)	Percentage (%)	Value (VND)	Percentage (%)
7	Imaging and Diagnostic Expenses	1,816,619,100	13.49	4,328,169,500	6.54
8	Blood Expenses	11,110,000	0.08	1,612,877,000	2.44
Total		**13,470,974,573**	**100**	**66,198,361,061**	**100**
2022					
1	Bed-day Expenses	0	0.00	10,285,916,250	13.80
2	Medication Expenses	10,478,990,560	56.79	15,184,837,418	20.38
3	Surgical Procedures Expenses	516,820,591	2.80	15,544,510,175	20.86
4	Laboratory Test Expenses	1,707,778,400	9.25	4,997,431,600	6.71
5	Medical Examination Expenses	2,788,169,250	15.11	59,704,000	0.08
6	Medical Supplies Expenses	2,567,168	0.01	21,663,133,607	29.07
7	Imaging and Diagnostic Expenses	2,952,374,200	16.00	4,935,050,200	6.62
8	Blood Expenses	6,006,000	0.03	1,844,269,000	2.48
Total		**18,452,706,170**	**100**	**74,514,852,250**	**100**

The highest rejection rate of medical expenses occurred in 2022 (Table 3). The rejection rate for inpatient health insurance medical expenses consistently exceeded that for outpatient health insurance medical expenses. The rejection rate for inpatient medical expenses remained stable at a constant rate of 0.3% from 2020 to 2022. However, the rejection rate for outpatient medical expenses fluctuated, increasing from 0.6% in 2020 to 0.8% in 2022.

Table 3. Rejection rate of health insurance medical expenses

Category	Total Number of Claim Requests	Requested Medical Expenses (VND)	Rejected Medical Expenses (VND)	Rejection Rate (%)
2020				
Inpatient	11,401	55,291,388,601	147,324,445	0.3
Outpatient	89,332	16,647,556,610	94,365,261	0.6
Total	*100,733*	*71,938,945,210*	*241,689,706*	*0.3*
2021				
Inpatient	10,180	54,722,144,429	175,330,987	0.3
Outpatient	56,341	11,731,596,348	34,306,582	0.3
Total	*66,521*	*66,453,740,777*	*209,637,569*	*0.3*
2022				
Inpatient	12,000	60,100,793,179	202,626,371	0.3
Outpatient	78,479	16,085,232,696	131,605,678	0.8
Total	*90,479*	*76,186,025,874*	*334,232,049*	*0.4*

The total value of rejected medical expenses increased from 241.7 billion VND in 2020 to 334.2 billion VND in 2022. Diagnostic imaging and functional exploration significantly increased from 13.3 million VNDs in 2020 to 30.4 million VNDs in 2022. Similarly, the number of errors related to medical orders sharply increased from 1.2 million VND in 2020 to 14.5 million VND in 2022. During the period from 2020 to 2022, the rejected expenses related to medication consistently accounted for the highest proportion (Table 4). Specifically, the use of non-listed medications has decreased annually; however, it remains the leading cause of drug rejection.

Table 4. Reasons for rejection of health insurance medical expenses

Rejection Reasons	2020		2021		2022	
	Value (VND)	Percentage (%)	Value (VND)	Percentage (%)	Value (VND)	Percentage (%)
Diagnostic Imaging - Functional Exploration	*13,297,000*	5.50	*16,503,000*	7.87	*30,357,594*	9.08
Errors related to medical orders	1,165,600	0.48	1,907,400	0.91	14,525,994	4.35
Errors related to the card (administrative information, registration location, period, etc.)	8,231,400	3.41	7,410,100	3.53	9,560,300	2.86
Incorrect pricing	2,805,000	1.16	2,964,300	1.41	1,362,700	0.41
Services not listed in the performed procedures	1,095,000	0.45	4,221,200	2.01	4,908,600	1.47
Surgery - Operation	*49,569,800*	20.51	*33,182,836*	15.83	*67,729,268*	20.26
Medical records using surgical fees more than once in a day	14,595,100	6.04	8,604,200	4.10	7,105,200	2.13
Errors related to the card (administrative information, registration location, period, etc.)	20,608,000	8.53	15,408,000	7.35	14,200,000	4.25
Errors related to medical orders	8,745,700	3.62	5,201,400	2.48	22,919,768	6.86
Incorrect pricing	5,621,000	2.33	3,969,236	1.89	23,504,300	7.03
Medication	*81,377,275*	33.67	*57,769,663*	27.56	*79,946,326*	23.92
Errors related to the card (administrative information, registration location, period, etc.)	3,700,778	1.53	2,109,400	1.01	406,800	0.12
Medication paid at a certain rate	3,659,811	1.51	2,304,800	1.10	39,025,549	11.68
Medication outside the approved list of drugs	70,356,875	29.11	52,406,300	25.00	33,798,178	10.11
Errors related to drug prices (higher than the approved price)	3,659,811	1.51	949,163	0.45	6,715,799	2.01
Bed fees	*43,810,500*	18.13	*46,188,600*	22.03	*52,106,250*	15.59
Payment of fees with a medical order date after the discharge date	9,225,900	3.82	4,301,500	2.05	4,520,200	1.35
Payment of bed days not in accordance with regulations (1 day bed larger than 4 hours)	4,160,000	1.72	2,160,000	1.03	1,565,288	0.47
Payment of bed days not in accordance with regulations (1 day bed smaller than 4 hours)	3,573,400	1.48	4,100,611	1.96	7,560,500	2.26
Payment of bed days not in accordance with regulations (outside special cases)	13,423,200	5.55	8,602,100	4.10	23,000,062	6.88

Rejection Reasons	2020		2021		2022	
	Value (VND)	Percentage (%)	Value (VND)	Percentage (%)	Value (VND)	Percentage (%)
Errors related to the card (administrative information, registration location, period, etc.)	12,253,800	5.07	3,552,300	1.69	15,460,200	4.63
Examination Fees	*15,050,700*	6.23	*13,480,950*	6.43	*38,914,809*	11.64
Medical records using a medical examination service more than once	2,622,500	1.09	3,169,648	1.51	6,210,200	1.86
Examination fee for more than 1 specialty incorrectly requested	4,990,150	2.06	3,650,210	1.74	6,320,500	1.89
Payment of fees with a medical order date after the discharge date	3,200,000	1.32	780,230	0.37	3,012,600	0.90
Errors related to the card (administrative information, registration location, period, etc.)	4,238,050	1.75	5,880,862	2.81	23,371,509	6.99
Medical Supplies	*16,817,675*	6.96	*18,964,320*	9.05	*27,674,802*	8.28
Errors related to the card (administrative information, registration location, period, etc.)	5,313,910	2.20	2,560,200	1.22	5,890,233	1.76
Medical supplies have a higher price than the approved price	6,200,000	2.57	10,356,200	4.94	9,038,369	2.70
Medical supplies outside the approved list of supplies used at the hospital	5,001,695	2.07	6,047,920	2.88	12,746,200	3.81
Laboratory Tests	*21,766,757*	9.01	*23,548,200*	11.23	*37,503,000*	11.22
Payment of fees with a medical order date after the discharge date	3,215,000	1.33	4,520,100	2.16	6,542,100	1.96
The distance between two tests is not in accordance with the regulations at Circular 35/2016/TT-BYT	8,412,200	3.48	9,865,200	4.71	9,500,200	2.84
Unreasonable test prescription	3,650,200	1.51	2,100,050	1.00	4,780,230	1.43
Errors related to the card (administrative information, registration location, period, etc.)	6,489,357	2.68	7,062,850	3.37	16,680,470	4.99
Total	241,689,706	100	209,637,569	100	334,232,049	100

DISCUSSION

During the period from 2020 to 2022, the proposed health insurance medical examination and treatment costs at hospitals accounted for the highest value in 2022, accounting for 81.9% of the total health insurance medical examination and treatment costs. This can be explained by various factors, including the impact of the COVID-19 pandemic and changes in the disease pattern, leading to increased healthcare costs [19–22].

Throughout Vietnam, especially in the years 2020-

2021, the influence of the COVID-19 pandemic resulted in an average of more than 145 million health insurance medical examinations and treatment visits per year. In 2022, the number of health insurance medical examinations and treatment visits exceeded 150 million, with the associated costs exceeding 105 trillion VND [23]. On the other hand, based on Decree 07/2021/ND-CP, more families were classified in the poor household group [24]. According to Decree 146/2018/ND-CP, individuals in poor households are eligible for 100% state budget support for health insurance card expenses [25]. Therefore, starting in 2022, more individuals were issued health insurance cards free of charge, contributing to the continuous increase in health insurance medical examination and treatment costs.

Additionally, since January 1, 2021, individuals with health insurance cards seeking medical treatment outside their designated area have received 100% reimbursement from the health insurance fund for provincial hospitals nationwide (compared to the previous 60% reimbursement). Hence, individuals with health insurance cards, when seeking medical treatment outside their designated area, enjoy 100% reimbursement for inpatient treatment nationwide. Patients can receive inpatient treatment in any province across the country, with the health insurance fund covering 100% of the medical examination and treatment costs based on the benefits of the health insurance card. This also contributes to the increase in total medical examination and treatment costs in 2022 [2],[10].

The process of health insurance assessment is a specialized activity conducted by the social insurance agency or in coordination with healthcare facilities to verify the procedures of health insurance medical examinations and treatments. It involves scrutinizing and evaluating the prescription of treatments and the use of medications, chemicals, medical supplies, medical equipment, and technical healthcare services for patients, according to regulations [26]. This assessment is crucial because it serves as the legal basis for the social insurance agency to settle medical examination and treatment expenses with healthcare facilities [2,26,27]. Our research indicates that, through health insurance assessment activities, the number of settlements has consistently been lower than the proposed costs from 2020 to 2022.

The highest rejected payment in 2022 was 334,232,049 VND, and all of it was a direct rejection from the assessment records. During the period of 2020-2022, the emergence and escalation of the COVID-19 pandemic, along with changes in health insurance policies and regulations, could also impact healthcare and assessment processes, affecting cost assessment and settlement procedures [28,29].

As reported in Quang Ngai Province (a province in Central Vietnam), unpaid costs due to exceeding the total payment during the period of 2019-2021 amounted to 117.959 billion VND. The Department of Health in Quang Ngai attributed this to the social insurance's application of an inappropriate total payment limit, which did not align with the actual amount the hospital had paid for the patient. The total settlement did not accurately reflect the actual demand for medical examinations and treatments at various units, resulting in settlements that are consistently lower than the actual health insurance medical examination and treatment costs [30].

According to reports from the Ministry of Health of Vietnam, health insurance assessment activities in Can Tho also face challenges. The acceptance or rejection of payment was sometimes deemed unreasonable because assessors rigidly enforced document regulations, and in practice, hospitals applied techniques and prescriptions that were suitable for patients. Moreover, the volume of assessment records was excessive (regulated at 30% in equivalent districts, approximately 7,000 records in Thoi Lai district, Can Tho), while there were only a limited number of assessors with medical expertise. Additionally, the assessment software was not fully developed, making it difficult to apply and process results with limited

effectiveness [31].

Over the years, from 2020 to 2022, the value of inpatient health insurance (HI) treatment costs has consistently been much greater than outpatient costs. In 2022, the value of inpatient treatment costs is four times greater than that of outpatient costs. This can be explained by changes in health insurance policies in Vietnam for inpatients. Specifically, starting in 2021, individuals with health insurance cards who seek inpatient treatment outside the designated facilities have expenses covered at a 100% reimbursement rate for provincial hospitals nationwide (compared to the previous 60% coverage by HI). This has led to an increase in the number of inpatient cases, and moreover, inpatients are predominantly those with multiple severe illnesses requiring prolonged treatment periods and incurring high costs for medical supplies and surgical procedures [2,10,32].

The global trend is toward strengthening outpatient treatment or providing healthcare services at lower levels, focusing on initial health care rather than prioritizing inpatient care [33,34]. This trend contrasts with the situation in Vietnam, where the proportion of patients receiving inpatient treatment is currently greater than 60%, while the proportion receiving outpatient treatment is only approximately 30%. Inpatients often come with family members for support, resulting in additional social costs [35].

The healthcare scenario in Vietnam is similar to that in China. From the perspective of healthcare services, there is an issue of excessive reliance on inpatient services. The reimbursement rate for outpatient care (20%) is much lower than that for inpatient care (90%), and some types of medication are only reimbursed when used for inpatient services. Consequently, patients in rural areas of China tend to prefer inpatient services, even if their conditions can be treated through outpatient visits [36].

For outpatient care, medication costs have been the most significant contributor, accounting for more than 50% of the total expenses over the years. In contrast, for inpatient health insurance (HI) expenses, medical supplies constitute the highest proportion, with medication costs also having a significant share. Our research on the reasons for payment rejection revealed that medication-related factors are the predominant reasons (from 2020 to 2022). Specifically, the use of non-listed drugs for HIs is common. In Vietnam, the drug list for HI has been slowly updated. Since 2018, the list of drugs within the scope of HI payments has not been comprehensively updated and supplemented with new drugs, as outlined in Circular 30/2018/TT-BYT [37,38]. The most recent update in Circular 20/2022/TT-BYT only supplemented the drug list for COVID-19 treatment without updating new drug categories [39]. This poses a challenge for hospitals because of the large number of patients seeking diagnosis and treatment and diverse illnesses requiring various medications. The drug list also plays a crucial role in influencing physicians' prescription decisions [40]. Additionally, since the COVID-19 pandemic, healthcare facilities have faced severe shortages of medications and medical supplies, affecting the healthcare activities of the population. To address this situation, in March 2023, the government continuously issued Decree No. 07/2023/NĐ-CP amending and supplementing provisions on the management of medical equipment and Resolution No. 30/NQ-CP on continuing to implement solutions to ensure drugs and medical equipment [41–43]. Regarding the drug list for HI, in 2023, the Ministry of Health of Vietnam drafted a plan that may be issued in the near future [44]. This contributes to addressing the reasons for payment rejections related to the use of drugs outside the prescribed list. In summary, the reimbursement of expenses for HI treatment requires coordination between healthcare facilities and social insurance agencies through the assessment of treatment costs. Identifying the reasons for payment rejection is crucial because it helps improve the treatment process and ensures compliance with HI regulations. The verification process should be transparent and fair to avoid disagreements and minimize cases of payment rejection. Furthermore, the Social Insurance Agency should share HI treatment data with management agencies such as the Ministry of Health to help

them develop better cost management policies, and hospitals can also check their data before submission to the Social Insurance Agency.

Limitations

This study has several limitations. The research team only investigated the situation of health insurance payment refusals at a hospital in Can Tho, Vietnam. Therefore, the results may not be representative of the entire healthcare system in Vietnam. The study was conducted during the period from 2020 to 2022, when the COVID-19 pandemic was still prevalent and significantly impacted the healthcare system. Since 2023, the COVID-19 pandemic has been controlled. In the 2020-2022 period, numerous legal documents related to medical examination and treatment activities and payment for medical examination and treatment expenses were issued and replaced each other, which could be a factor leading to inaccuracies in the research. Moreover, the study did not delve deeply into specific drug categories to analyze the causes of payment refusals more comprehensively. This aspect could be explored in more detail in future studies.

CONCLUSION

Based on the research results during the 2020-2022 period, it is evident that from 2020 to 2022, the settled amounts were consistently lower than the proposed expenses. The expenses for inpatient health insurance were more frequently denied than those for outpatient health insurance, and the main reason for payment refusal remains focused on costs related to medication, particularly the use of drugs outside the approved list. This poses challenges in cost management, necessitating improvement measures and solutions to address the issue of payment refusals within the health insurance system.

Duyen Thi My Huynh and Van Nong Lai contributed equally to this work.

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No.

CONFLICTS OF INTEREST

Not applicable

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: DTMH, VNL, VDT; Methodology: DTMH, TTL, VDT, QLDV; Investigation: DTMH, TTL, HVN; Resources: DTMH, VNL, TTL, THYN; Writing – original draft: DTMH, VNL, TTL, HVN, THYN, QLDV, VDT; Writing – review & editing: DTMH, VNL, TTL, HVN, THYN, QLDV, VDT.

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استكشاف رفض دفع تكاليف الرعاية الصحية المغطاة بالتأمين الصحي: دراسة حالة لمستشفى في فيتنام

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ملخص

يشكل الفصل في المطالبات ورفض دفع تكاليف الرعاية الصحية المغطاة بالتأمين الصحي تحديات في الحفاظ على تشغيل المستشفيات وضمان حقوق المرضى والطاقم الطبي .لا تزال الأبحاث حول هذه القضية محدودة على الصعيدين العالمي وفي فيتنام .تهدف هذه الدراسة إلى فهم الوضع الحالي لرفض دفع تكاليف الفحوصات الطبية والعلاجات المغطاة بالتأمين الصحي في أحد مستشفيات كان ثو، فيتنام، واستكشاف أسباب رفض الدفع .استخرجت دراسة وصفية مقطعية البيانات المتعلقة بالنفقات الطبية المغطاة بالتأمين الصحي والتي تم رفضها في مستشفى في كان ثو، فيتنام، على مدى ثلاث سنوات من 1يناير 2020إلى 31ديسمبر .2022أظهرت الدراسة أنه بين عامي 2020و2022، كان عدد المدفوعات المسواة أقل باستمرار من التكاليف المقترحة .وبلغ أعلى مبلغ مرفوض في عام 2022حوالي 334,232,049دونغ فيتنامي .(0.4%)واجهت نفقات التأمين الصحي للمرضى الداخليين معدل رفض دفع أعلى مقارنة بنفقات المرضى الخارجيين . كانت الأدوية، والإجراءات الجراحية، ورسوم الإقامة في المستشفى هي الأسباب الثلاثة الرئيسية لرفض الدفع بين عامي 2020و .2022يظل السبب الرئيسي لرفض الدفع مرتبطًا بتكاليف الأدوية، مما يشكل تحديات في إدارة التكاليف ويستدعي اتخاذ تدابير لمعالجة هذه المشكلة داخل نظام التأمين الصحي.

الكلمات الدالة: التأمين الصحي، تكلفة الأدوية، مقدم الرعاية الصحية، المستشفى، فيتنام.

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Diclofenac Adsorption from Contaminated Water onto Olive-Leaf-Derived Adsorbent

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ABSTRACT

This study investigates the adsorption of diclofenac (DCF) onto an olive leaf-derived adsorbent. The harvested olive leaves were washed, dried, and powdered then extracted with 80% ethanol. The extraction was filtered, washed with sodium hypochlorite, and ethanol, and then dried. The material was then activated using sodium hydroxide, phosphoric acid, and dead sea water, for the adsorption of DCF from contaminated water being investigated. Various operational parameters such as dosage, contact time, DCF concentration, and pH were systematically varied to understand their influence on adsorption efficiency. The kinetics of DCF adsorption followed pseudo-second-order kinetics. Isothermal studies revealed that the adsorption process conforms well with the Freundlich isotherm, suggesting multilayer adsorption onto a heterogeneous surface. Thermodynamic analysis indicated that the adsorption process is spontaneous and exothermic. Morphological analysis completed using the SEM data demonstrated a transformation in the porous structure of the adsorbent, indicating effective pore occupation by DCF molecules post-adsorption. Overall, the results demonstrate the effectiveness of olive leaf-derived adsorbent in efficiently removing DCF from aqueous solutions.

Keywords: Contamination; Emerging Contaminant; Thermodynamics; Water Treatment; Adsorption.

1. INTRODUCTION

Pollution of water by pharmaceutical chemicals has been a major environmental concern in recent years [1]. One of the widely used pharmaceutical medications of interest is diclofenac (DCF), the most-selling nonsteroidal drug known for its pain-relieving and anti-inflammatory properties [2, 3]. Many studies including [4] and [5] have found DCF in water bodies. [6] found DCF in 83% of European rivers' water samples. [7] detected DCF in China's Huangpu and Beiyun rivers. In Taiwanese waterways, [8] found DCF in 23 different sites. Several studies have also found DCF in water bodies in USA, Korea, Spain, Serbia, Greece, and Portugal, as reported by [9-15].

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The presence of DCF in water can be toxic to both humans and several aquatic organisms, including bacteria, algae, fish, and microcrustaceans [16, 17]. DCF's presence in many water bodies across the world can be related to its widespread use as a painkiller and anti-inflammatory medicine and its resistance to degradation using typical treatment conditions. Only about 60% of DCF can be eliminated from water using traditional treatment methods[18-20].

Currently, there is a need to develop more advanced and effective technologies to remove DCF from water systems. Some promising methods include ozonation[21], membrane filtration[22], advanced oxidation techniques [23], Fenton oxidation[24], and adsorption[25]. Several of the aforementioned methods often come with various complications including intricate procedures, high expenses, and the management of toxic sludge[26]. However,

adsorption remains a favored choice due to its simplicity, efficiency, and cost-effectiveness [26, 27].

Many materials are available for DCF adsorption from contaminated water including chemically activated cocoa pod husks[28], activated carbon derived from sewage sludge[29], graphene oxide[30], multiwalled carbon nanotubes[31], commercial organoclay[32], and activated carbon from tea waste[33].

Jordan is one of the main olive producing countries in the world and harvests approximately 296,814 tons of olives every year [34]. In the harvesting season, significant volumes of leaves are generated as waste. [35] introduced an adsorbent derived from olive leaves for capturing lead, a highly toxic heavy metal, from polluted water. The objective of this study is to investigate the efficacy of the olive-leaf-derived adsorbent for removing DCF from contaminated water. The effect of several adsorption parameters such as adsorbent dosage, contact time, initial DCF concentration, solution pH, and temperature were investigated. The experimental data are used to develop an equilibrium isotherm and also a kinetic model for predicting the adsorption process.

2. MATERIALS AND METHODS

2.1. Materials

During the olive harvesting season in 2022, fresh olive tree leaves were gathered from a farm located in Irbid, Jordan. Dead Sea water was brought from the Dead Sea, Jordan. The diclofenac (DCF) used in the study was obtained from Dar Al-Dawa Industrial Company, Amman-Jordan. Pure sodium hydroxide pellets (with a purity of 98.5%) were procured from Fisher Scientific International, UK. Jordan Chemical Industries supplied the sodium hypochlorite. Sigma Aldrich supplied acetonitrile with a purity of 99.9%. Jordan Phosphate Mines Company provided the phosphoric acid.

2.2. Preparation of the olive-leaf-derived adsorbent

Freshly harvested olive leaves underwent thorough washing with distilled water to remove stuck dirt, followed by controlled air-drying in a dark condition. The dried leaves were then finely powdered using a grinder and subjected to extraction following the procedure detailed by [36]. The procedure encompassed combining 200 g of powdered leaves with 500 mL of 80% ethanol extraction solvent in a volumetric flask, followed by 5 hours of agitation to facilitate efficient extraction of plant constituents. The extracted material was kept in the room until reaching lab temperature and then filtered using a Whatman No.42 filter in order to obtain fine particles only. Soaking the fine sample with 5 L of sodium hypochlorite and filtering resulted in a white creamy fine sample (the process was repeated about fives times). The white creamy sample was cleaned from the sodium hypochlorite when it was washed with 80% ethanol and distilled water. The clean sample was dried in an oven at 70°C. The obtained dry sample was activated following the procedure introduce by [37] and [35]. The method involves combining 10 g of the sample with 3.5 g sodium hydroxide and 2 mL phosphoric acid along with progressively adding 10 mL of Dead Sea water with stirring for 15 minutes. The obtained product was then cleaned with distilled water and dried in an oven at 70°C. The applicability of using the obtained dry material in the adsorption of DCF from contaminated water was investigated in this study.

The abundance of valuable salts in Dead Sea water is crucial in the activation method to enhance the adsorption capacity. Where the active binding sites in the biomass are transformed from H+ to Ca+, Na+, Mg+, and K+ with the help from using phosphoric acid and sodium hydroxide [35, 37].

2.3. Experimental studies

The Phenom XL G2 SEM was used to study the surface structure of the olive-leaf-based adsorbent. SEM was performed in high vacuum with a 15 kV accelerating voltage to improve image quality. The samples were conductive carbon taped on SEM stubs and platinum-sputter-coated before imaging. Samples were mounted on SEM stubs with conductive carbon tape and sputter-coated with platinum before imaging. This preparation increased

electrical conductivity and reduced imaging charging.

All adsorption studies used 10 mL DCF-contaminated solutions. Testing samples were placed in a temperature-controlled water shaker bath at 100 rpm. After treatment, DCF concentrations were determined using Thermo-Electron Corporation HPLC at 280 nm and 1 mL/min.

Several experiments were conducted to determine how various operational parameters affect DCF adsorption. To study how the operational parameters affect absorptiometry, the adsorbent dose (5–80 mg/mL), initial DCF concentration (0.04–1 mg/mL), contact time (5–120 minutes), and solution pH (6–11 pH) were varied systematically. DFC removal percentages were estimated using Equation 1 presented in Table 1. Where C_i and C_f are DCF concentrations at the beginning (initial) and end (final) of the adsorption (mg/mL), respectively.

Table 1. Adsorption removal percentage, capacity, isotherms, and kinetics governing equations utilized in this study

Equation*	Reference	Equation sequence
$\%\ DCF\ removal = (C_i - C_f)/C_i \times 100\%$	[38]	(1)
$C_e/q_e = 1/q_{max}b + C_e/q_{max}$	[39]	(2)
$log\ q_e = logK_f + (1/n)\ logC_e$	[40]	(3)
$q_e = \left[(C_i - C_f)V\right]/M$	[35]	(4)
$log\ (q_e - q_t) = logq_e + (K_1/2.303)\ t$	[41]	(5)
$t/q_t = 1/(K_2 q_e^2) + (1/q_e)\ t$	[42]	(6)
$q_t = [(C_i - C_t)V]/M$	[28]	(7)
$K_L = q_e/C_e$	[43]	(8)
$\Delta G^o = -RT \ln K_L$	[44]	(9)
$\ln K_L = (\Delta S^o/R) - (\Delta H^o/(RT))$	[45]	(10)

* The symbols in Table 1 are all defined in the text at their first occurrence.

The adsorption isotherm was investigated by conducting experimental tests y varying C_i (from 0.04 to 1 mg/mL) while ensuring the attainment of the equilibrium condition with constant values for the remaining parameters. The obtained equilibrium data were subjected to fitting using the two commonly employed models, Langmuir (Equation 2 in Table 1) and Freundlich (Equation 3 in Table 1) isotherms. Where q_e is the adsorption capacity at equilibrium (mg/g) which can be calculated using Equation 4, q_{max} is the maximum adsorption capacity (mg/g), b is the Langmuir constant (L/mg), K_f is the Freundlich constant that represents the measure of the adsorption capacity ((mg/g) $(L/mg)^n$), n represents the adsorption intensity parameter, V is the volume of the solution (mL), and M is the mass of the olive-leaf-derived adsorbent (g).

The kinetics of DCF adsorption onto the olive leaf-derived adsorbent were investigated using pseudo-first-order (Equation 5 in Table 1) and pseudo-second-order (Equation 6 in Table 1) kinetics models. Where K_1 and K_2 are the adsorption rate constant for pseudo-first-order and pseudo-second-order kinetic models, respectively, q_t is the adsorption capacity at time t which can be estimated using Equation 7 (Table 1).

Thermodynamic parameters for the adsorption of DCF onto the olive leaf-derived adsorbent were determined through experimental tests conducted at different temperatures (from 25 to 45 ºC), while keeping all other parameters constant. These thermodynamic parameters encompass the Gibbs free energy change (ΔG^o), the enthalpy change (ΔH^o), and the entropy change (ΔS^o), providing valuable insights into the adsorption process.

Equation 8-10 (Table 1) were used to estimate the thermodynamic parameters. Where K_L is the equilibrium adsorption constant, R is universal gas constant, and T is the temperature (in Kelvin).

3. RESULTS AND DISCUSSION

3.1. Scanning electron microscopic analysis

The morphology of the olive leaf-derived adsorbent reveals a highly porous structure (Figure 1A). The SEM images of the olive leaf-derived adsorbent depict a notable transformation from a porous structure before DCF adsorption (Figure 1A) to a seemingly filled appearance post-adsorption (Figure 1B), indicating significant pore occupation by DCF molecules. This observation suggests a robust adsorption mechanism where DCF molecules effectively penetrated the pore structure of the adsorbent to occupy available surface sites.

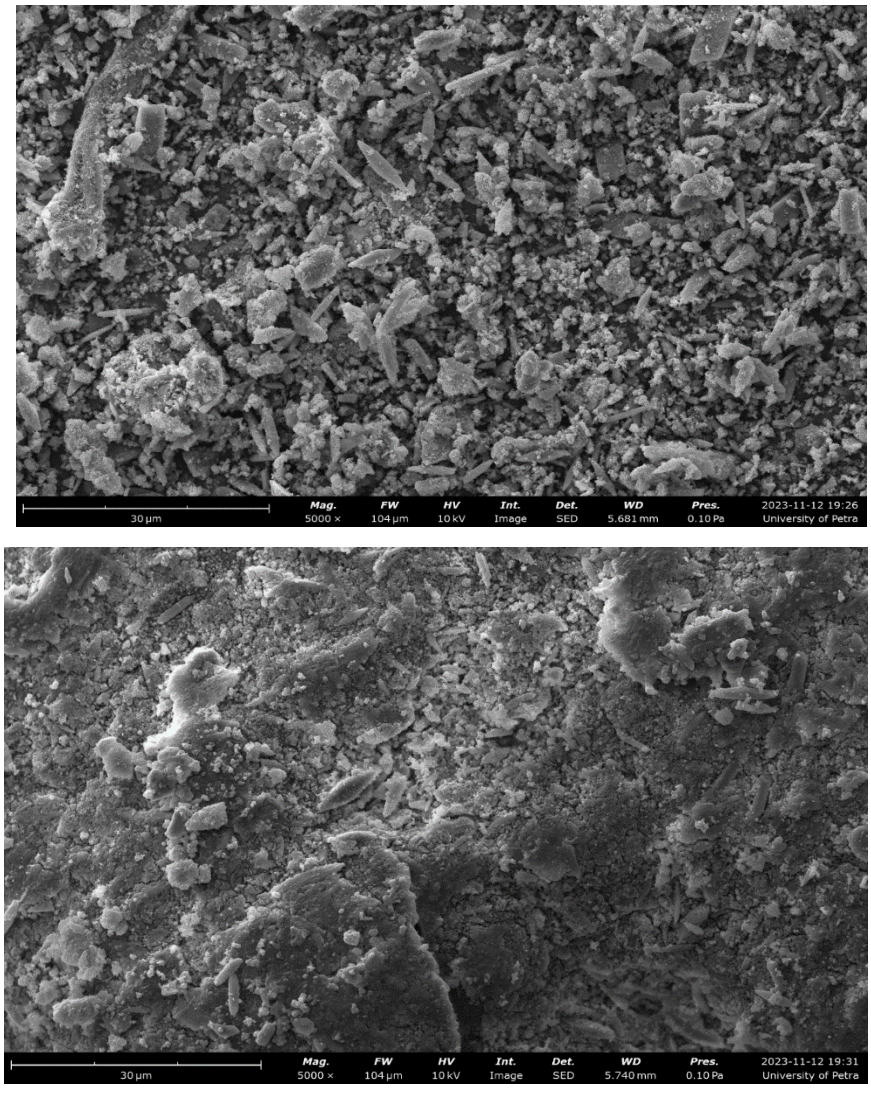


Figure 1. SEM images of the olive-leaf-derived adsorbent (A) before adsorption and (B) after adsorption

3.2. Effect of adsorbent dose

The adsorption of DCF (concentration = 1 mg/mL) was performed using the olive-leaf-derived adsorbent across a range of doses (5 to 80 mg/mL) over 120 minutes at a pH of 6. Initially, at 5 mg/mL, DCF removal was only around 8%, whereas at 80 mg/mL, it dramatically increased to 89% (Figure 2). This notable improvement in removal percentage with higher doses can be attributed to the increased availability of active sites on the adsorbent surface [46].

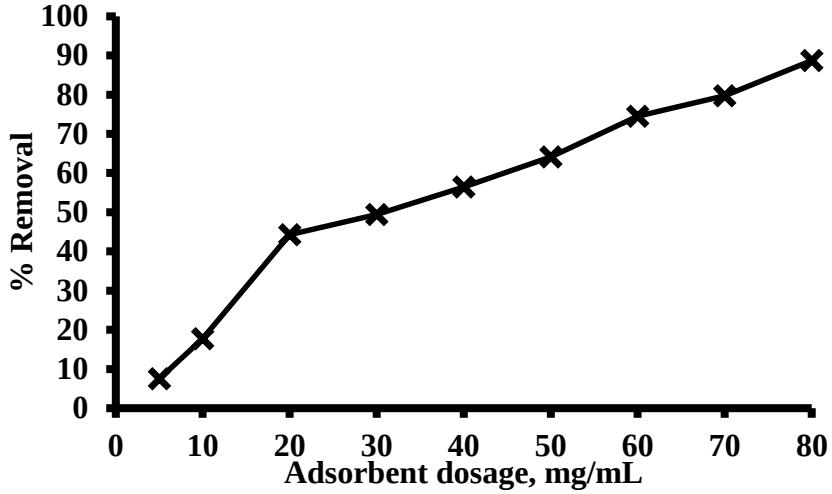


Figure 2. Effect of the olive-leaf-derived adsorbent dosage on DCF adsorption removal percentage

3.3. Effect of contact time

To establish the equilibrium time required for DCF adsorption (initial concentration = 1 mg/mL), experiments were conducted over a time range of 5 to 120 minutes, using a constant adsorbent dosage of 80 mg/mL. Figure 3 depicts the observed trend, showing a steady increase in uptake with time until equilibrium was reached at 60 minutes. Notably, no further uptake was recorded beyond this duration. The decline in adsorption over time can be attributed to the depletion of available adsorption sites [47].

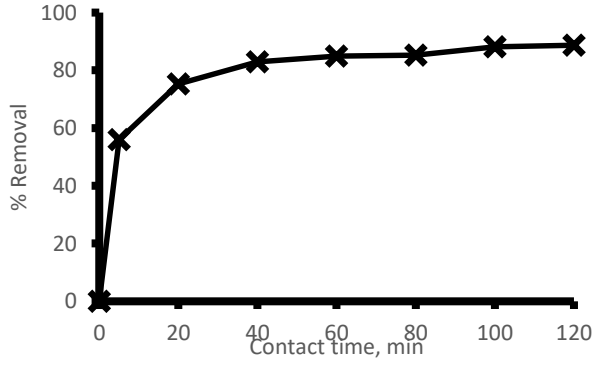


Figure 3. Effect of contact time on DCF adsorption removal percentage onto the olive-leaf-derived adsorbent

3.4. Effect of solution pH

The experimental findings presented in Figure 4 demonstrated the highest removal percentage of DCF at pH 6, reaching approximately 88.7%. As the pH increased from 7 to 11, there was a slight decrease in the removal percentage, although remaining relatively high (stabilizing around 83%). This trend suggests that within the tested pH range (6 to 11), the adsorption of DCF onto the olive leaf-derived adsorbent is consistently effective, with slightly enhanced efficiency observed at lower pH values. The pH levels below 6 were not examined because DCF is only slightly soluble in water under acidic conditions [48].

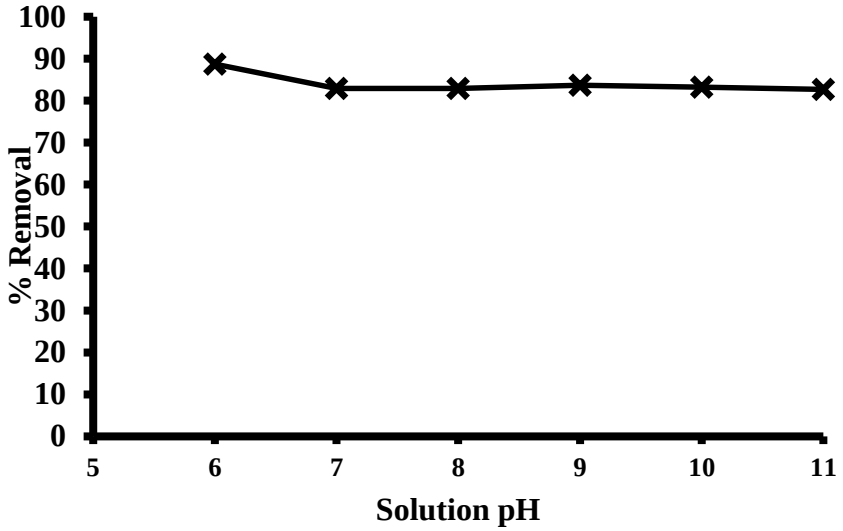


Figure 4. Effect of Solution pH on the percentage of DCF adsorption removal using olive leavf-derived adsorbent (Adsorbent dose = 80 mg/mL; contact time = 120 min)

Effect of DCF initial concentration

Figure 5 presents the outcomes of an experimental investigation aimed at evaluating the influence of varying initial concentrations of DCF (0.04 to 1 mg/L) on its removal efficiency using the olive-leaf-derived adsorbent. As initial DCF levels increased, removal percentages declined. The peaked removal percentage of 88.7% was obtained at an initial concentration of 0.04 mg/mL, which was decreased to 68.41% at 1 mg/mL. The increase in DCF concentration reduces the available adsorption sites, which may explain the decline in removal percentages [33, 49, 50].

3.5. Adsorption kinetics

The investigation of the adsorption kinetics data was carried out using the pseudo-first-order and pseudo-second-order models. The linearized forms of these two models were used, which are presented in Equations 5 and 6 in Table 1. Results presented in Figure 6 show that both models represent the kinetics data of DCF adsorption onto the olive-leaf-derived adsorbent well. A summary of both models' parameters are presented in Table 2. For the pseudo-first-order model, the computed equilibrium adsorption capacity (q_e) and rate constant (K_1) were 6.03 mg/g and 3.7×10^{-2} 1/min, respectively, and the estimated R^2 value was 92.2 %, indicating a good correlation with the experimental data. Conversely, the pseudo-second-order model showed a lower q_e of 0.456 mg/g, a rate constant (K_2) of 0.591 g/(mg.min), and an excellent match to the experimental data (R^2 = 99.9 %). The higher R^2 value of the pseudo-second-order model indicates a more accurate representation of the adsorption kinetics.

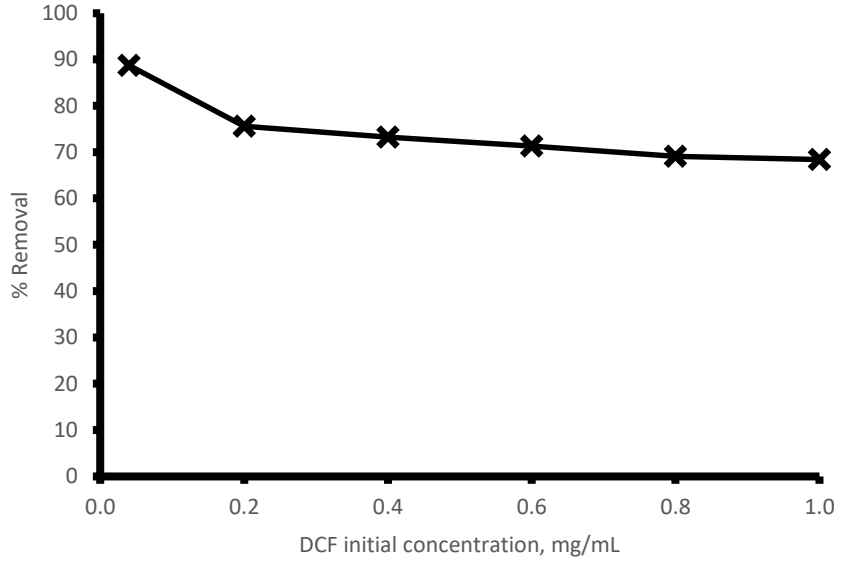
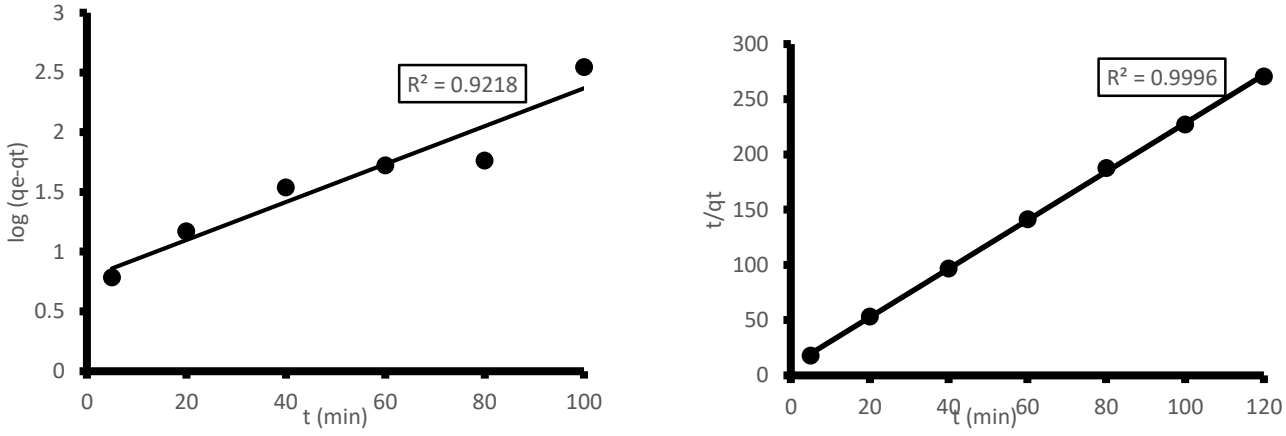


Figure 5. Effect of initial DCF concentration on the percentage of DCF adsorption removal by olive leaf-derived adsorbent



A **B**

Figure 6. Fitting of adsorption kinetic data for DCF adsorption onto the olive leaves-derived adsorbent using: (A) pseudo-first-order and (B) pseudo-second-order

Table 2. Kinetic parameters for DCF adsorption onto olive-leaf-derived adsorbent

Kinetics parameters and R^2	
pseudo-first-order model	
q_e (mg/g)	6.03
K_1 (1/min)	3.7×10^{-2}
R^2	92.2%
pseudo-second-order model	
q_e (mg/g)	0.456
K_2 (g/(mg.min))	0.591
R^2	99.9%

3.6. Adsorption isotherms

The fittings of the isotherm experimental data were compared against both Langmuir and Freundlich models. The Freundlich model goodness of fit (R^2 = 99.5%) demonstrated superior fitting compared to the Langmuir model (R^2 = 76.1 %). Figure 7 demonstrates that the Freundlich isotherm offers a more precise depiction of the experimental data.

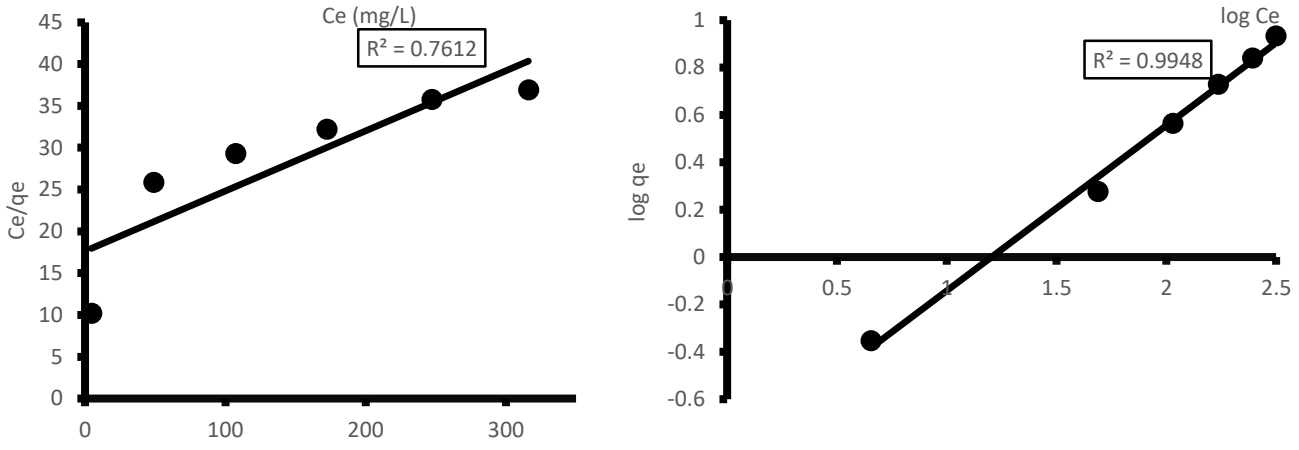
The Freundlich isotherm, defined by the maximum adsorption capacity (Q_{max}) and the Langmuir constant (K_L), models monolayer adsorption on homogeneous surfaces [51, 52]. As reported in Table 3, the Langmuir constant K_L was found to be 0.00408 and the maximum adsorption capacity Q_{max} was found to be 14 mg/g. The low R^2 value of (76%) suggests that there are deviations from monolayer adsorption or surface homogeneity. Lower R^2 values prompted inquiries regarding the suitability of the Langmuir model in completely elucidating the adsorption of DCF on the adsorbent derived from olive leaves.

The Freundlich isotherm, defined by the Freundlich constant (K_f) and the exponent (n), models multilayer adsorption on heterogeneous surfaces [35, 43, 53]. The high R^2 value of 99.5% suggests a robust correlation with the experimental data. The Freundlich exponent (n) of 1.434 suggests favorable adsorption, as values between 1 and 10 typically indicate good adsorption [54]. The Freundlich constant (K_f) of 0.1449 represents the adsorption capacity (Table 3).

In summary, the Freundlich isotherm model appears to be more appropriate for describing the adsorption of DCF onto the olive-leaf-derived adsorbent, indicating multilayer, heterogeneous adsorption under favorable conditions.

3.7. Thermodynamic parameters

The plot of ln (K_L) versus 1/T (Figure 8) shows a linear relationship, suggesting that the adsorption process follows the Van't Hoff equation. The slope and intercept from Figure 8 were used to calculate the values of the enthalpy (ΔH^o) and entropy (ΔS^o). The obtained negative values for both ΔH^o and ΔS^o (Table 4) indicated that the adsorption process of DCF onto the olive-leaf-derived adsorbent is exothermic and associated with a decrease in randomness. The calculated Gibbs free energy (ΔG^o) values (Table 4) were also negative at all temperatures, indicating that the adsorption process is thermodynamically favorable and spontaneous [55].



A **B**

Figure 7. Fitting of equilibrium data for DCF adsorption onto the olive leaf-derived adsorbent to the isotherm models. (A) Langmuir isotherm and (B) Freundlich isotherm

Table 3. Isotherm parameters for DCF adsorption onto olive-leaf-derived adsorbent

Isotherm models	isotherm parameters and regression coefficients	
	Q_m (mg/g)	14
Langmuir isotherm	K_L (L/mg)	0.00408
	R^2	76.1%
	K_f (mg/g).(L/mg)^n	0.1449
Freundlich isotherm	n	1.434
	R^2	99.5%

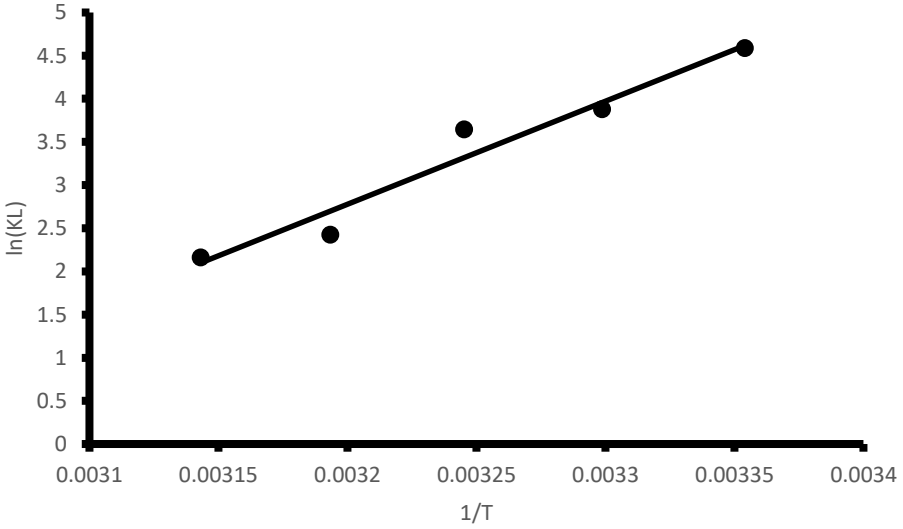


Figure 8. The plot of ln (K_L) versus 1/T for determining thermodynamic parameters of DCF adsorption on the olive-leaf-derived adsorbent

Table 4. Thermodynamic parameters for the adsorption of DCF onto the olive-leaf-derived adsorbent.

ΔH^o (kJ/mol)	ΔS^o (J/mol.K)	ΔG^o (kJ/mol)				
		Temperature (K)				
		298	303	308	313	318
-99.299	-0.295	-11.37	-9.77	-9.34	-6.32	-5.72

4. CONCLUSIONS

Our investigation examined the adsorption behavior of diclofenac (DCF) onto olive leaf-derived adsorbent. We explored the influence of adsorbent dosage, contact time, initial DCF concentration, and pH on adsorption efficiency. Our data show that 80 mg/mL adsorbent dose, 120 min contact time, and 0.04 mg/L DCF initial concentration are the optimal conditions for maximum removal. Kinetic studies demonstrated rapid uptake, which was well represented by pseudo-second-order model. The isotherm analysis highlighted Freundlich isotherm is the best option for describing the sorption reaction. The results of the thermodynamic analyses confirmed the spontaneous, exothermic nature of the sorption process. The study shows olive-leaf-derived absorbents can be extremely useful for treating DCF-contaminated water. Future research might be interesting to focus on developing pilot tests to evaluate the feasibility and cost-effectiveness in large-scale applications.

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امتزاز الديكلوفيناك من المياه الملوثة على مادة مازة مشتقة من أوراق الزيتون

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ملخص

تبحث هذه الدراسة في امتزاز الديكلوفيناك (DCF)على مادة مازة مشتقة من أوراق الزيتون. تم غسل أوراق الزيتون التي تم جمعها وتجفيفها وطحنها إلى مسحوق، ثم استخلاصها باستخدام الإيثانول (بنسبة نقاوة 80%). بعد ذلك، تم ترشيح المستخلص وغسله بهيبوكلوريت الصوديوم والإيثانول ثم تجفيفه. تم تنشيط المادة باستخدام هيدروكسيد الصوديوم وحمض الفوسفوريك ومياه البحر الميت لتعزيز قدرتها على امتزاز الديكلوفيناك من المياه الملوثة قيد الدراسة. تم دراسة تغيير عدة عوامل تشغيلية بشكل منهجي، مثل جرعة المادة المازة ووقت التلامس وتركيز الديكلوفيناك ودرجة الحموضة (pH)، لفهم تأثيرها على كفاءة الامتزاز. أظهرت دراسة حركية الامتزاز أن العملية تتبع نموذج حركية الامتزاز من المرتبة الثانية. كشفت دراسة إيزوثيرمات الامتزاز أن عملية الامتزاز تتبع نموذج فريندليش، مما يشير إلى حدوث امتزاز متعدد الطبقات على سطح غير متجانس. أظهرت الدراسة الثرموديناميكية للامتزاز أن عملية الامتزاز تلقائية وطاردة للحرارة. وأكد التحليل المورفولوجي باستخدام المجهر الإلكتروني الماسح (SEM)حدوث تغييرات في البنية المسامية للمادة المازة، مما يدل على الامتلاء الفعّال للمسام بجزيئات الديكلوفيناك بعد الامتزاز. بشكل عام، توضح النتائج فعالية المادة المازة المشتقة من أوراق الزيتون في إزالة الديكلوفيناك بكفاءة من المحاليل المائية.

الكلمات الدالة: التلوث؛ الملوثات الناشئة؛ الدراسة الثرموديناميكية؛ معالجة المياه؛ الامتزاز.

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Evaluation of the Therapeutic Effect of Cardamom Extract on Nephropathy Induced by Aspirin in Rats Model

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ABSTRACT

Analgesic-induced nephropathy is a serious complication resulting from the chronic overuse of analgesics, especially over-the-counter drugs such as aspirin. The study aimed to investigate the therapeutic effect of cardamom extract on aspirin-induced nephropathy in rat models. Twenty-four female Albino Wistar rats were randomly divided into three groups (n=8/group): control(no treatment); (ASA) aspirin 600 mg/kg/day for 4 days, and (ASA + Card) aspirin + cardamom extract 200 mg/kg/day for 7 days. Cardamom aqueous extract was prepared. Phenol and flavonoid contents were calculated. The kidney/body weight ratio was calculated, and serum urea and creatinine were measured. Lipid peroxidation was evaluated by measuring Malondialdehyde (MDA) concentrations, and superoxide dismutase (SOD) activity in kidney tissue. Histological alterations were also assessed. Parametric data were evaluated using the One-way analysis of variance (ANOVA) test, followed by Tukey's test. Nonparametric data were evaluated by the Mann–Whitney test and Fisher's tests. The results were considered significant at $P<0.05$. Total phenol 23.4 mg gallic acid equivalents / g dry extract, and flavonoids 1.77 mg quercetin equivalents /g dry extract. In the ASA group, kidney weight/body weight ratio, Serum biomarkers, and MDA concentrations were significantly increased, while SOD levels decreased, compared with the control group. The histological examinations showed significant tubular and glomerular injuries. There was a significant improvement in the Card histological and serum when compared with the aspirin group. Cardamom aqueous extract (200 mg/kg) showed effective therapeutic ability against aspirin-induced nephropathy by improving kidney functions, and enzymatic and histological parameters, due to their antioxidant activity in oxidative stress induced by aspirin.

Keywords: Kidney; Nephropathy; Nonsteroidal Anti-Inflammatory Drugs; Aspirin; Cardamom Extract; Oxidative Stress.

INTRODUCTION

Aspirin (acetylsalicylic acid) is a nonsteroidal anti-inflammatory drug (NSAID) that has antiplatelet, anti-inflammatory, antipyretic, and analgesic effects in the treatment of rheumatoid arthritis and prevention of thrombotic cardiovascular disease [1]. Long-term therapeutic administration of aspirin is associated with its harmful effects on the kidney, which can lead to renal disorders. Aspirin also initiates oxidative stress in the kidney by inhibiting the activity of antioxidant enzymes in mitochondria [2]. It also increases the production of reactive oxygen species (ROS), increasing the possibility of apoptosis and necrosis within the kidney cells [3], and that ends with many pathological effects such as tubular necrosis and tubulointerstitial nephritis [4]. Recently,

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interest has increased in natural antioxidants from medicinal plant sources as a more acceptable and safe choice than chemical ones [5]. The previous studies focused on plants that have high contents of polyphenols (phenols and flavonoids), which are secondary metabolites with significant antioxidant activity [6,7]. It is essential to focus on therapeutic options for oxidative stress-induced diseases that have not been commonly used before, such as cardamom, one of the most widely available and tolerable medicinal plants.

Cardamom (Elettaria cardamomum (L.) Maton) is a perennial herbaceous plant belonging to the Zingiberaceae family and grows in India, Guatemala, Sri Lanka, Nepal, and Indonesia. It is the third most expensive spice after saffron and vanilla. Cardamom is a traditional medicine used for several diseases like flatulence, bronchitis, asthma, kidney disorders, arthritis, congestion, and itching. Cardamom is a rich source of antioxidant components of phenolic compounds like quercetin, kaempferol, and luteolin that can suppress free radicals activity by preventing oxidation reactions, thus leading to reducing cellular injury [8]. Previous studies investigated the antioxidant properties of cardamom extracts either through protective effects in various oxidative stress-related diseases [9] or through therapeutic effects [10]. Cardamom almost reverses all the mechanisms by scavenging the formation of free radicals due to its high contents of flavonoids [11].

This study investigates the possible therapeutic effects of aqueous cardamom in preventing aspirin-induced nephropathy in rats.

MATERIALS AND METHODS

2.1 Animals

Twenty-four adult female Wister albino rats weighing (100-200) g were obtained from the animal breeding colony of the Atomic Energy Commission of Syria, Damascus, Syria. The rats were maintained in clean plastic cages and provided ad libitum and water. The laboratory animal room was under controlled environmental conditions (temperature 23±2°C, humidity 55±15%, 12-hour light/dark cycle), and the animals were acclimatized for one week before the start of experiments. All procedures in this study conformed to the guiding principles for research-involving animals and were approved by the Research Ethical Committee of the Faculty of Pharmacy, Damascus University, and conducted according to the guidelines of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals.

2.2 Experimental design

The rats were randomly divided into three groups (8 rats in each group):(Control): no treatment; (ASA): aspirin-CMC suspension (600 mg/kg/day P.O) at 24 h intervals for four days [12]. (ASA + Card): Cardamom aqueous extract (200 mg/kg P.O) beginning 24 h after the fourth aspirin dose at 24 h intervals for seven days [13]. At the end of the experimental period, the animals from each group were sacrificed. Blood samples were collected through heart puncture and serum was separated for renal function tests (BUN and creatinine). The kidneys were excised and weighed, and the macroscopic change was checked. One kidney was stored at −80°C and then homogenized for oxidative stress biochemical analysis (MDA, SOD), while the other one was sectioned longitudinally into two equally sized pieces and fixed in a 15% buffered formalin solution for histopathological studies.

2.3 Drugs

Aspirin (ASA): (The global company for pharmaceutical industries, Unipharma).

2.4 Plant material

The dried Cardamom fruits (E. cardmomum) were obtained from Al-Attar Herbal Pharmaceutical Industries (Damascus, Syria) and ground into a fine powder to make the aqueous extract.

2.5 Preparation of aqueous extract

Twenty grams of cardamom fine powder were added

to 100 ml distilled water, mixed well, and then kept at room temperature for 24 h on a magnetic stirrer with 2500 rpm. The mixture was filtered using filter paper. The resulting filtrate was centrifuged at 5000 rpm for 15 min, and the supernatant was filtered. The concentration of the filtrate (aqueous extract) was adjusted with distilled water to reach a final concentration of 200 mg/mL and collected in sterilized test tubes cotton plugged and stored in a refrigerator until ready to use [14,15]. The extract showed an extractive yield (7%).

2.5.1 Total phenol content determination

The total phenol contents of aqueous extracts were determined based on the Folin-Ciocalteu method described by [16], with some modulations. 20 µL of the aqueous extract was mixed with 1.58 ml of distilled water and then added to 100 µL of Folin-Ciocalteu reagent. 300 µL of 20% sodium carbonate was added after 5 minutes. The resulting greenish-blue solution was incubated in a dark place at room temperature for 2 hours. The absorbance was measured at 765 nm. Gallic acid was used as a calibration curve standard, using five serial dilutions (0, 100, 200, 250, 300, 400, and 500 mg/L) in methanol, which was used as a blank, and each reading was triplicated. The total phenol content was expressed as milligrams of gallic acid equivalent (GAE) per g of extract.

2.5.1 Total flavonoid content measurement

Total flavonoids in the aqueous extracts were determined by the aluminum chloride colorimetric method as mentioned by [17] with few modifications. 2 ml of aqueous extract mixed with 0.1 mL aluminum chloride (10%) and 0.1mL sodium acetate (1M) and then with 2.8 ml of distilled water. The absorbance was taken at 415 nm after incubation for 30 min at room temperature. Quercetin was used as a standard to construct the calibration curve using six different concentrations (0, 2.5, 5, 10, 15, 20µg/mL) in methanol solvent. The estimation of total flavonoids in the extracts was triplicated, and the results were averaged. The concentrations were expressed as milligrams of quercetin equivalent per g of extract.

2.6 Kidney weight/body weight ratio (%)

The rats were checked for food intake and body weight at regular time intervals from day 0 to day 11 to calculate the body weight change ratio (%). Kidney weight/ body weight change ratio (%) was also calculated.

2.7 Serum Creatinine Concentration

Serum creatinine levels were estimated by an assay Kit (Biosystems Company). The creatinine of the sample reacts with picrate in an alkaline medium and forms a colored complex that was measured spectrophotometrically at 500 nm, and the concentrations were calculated accordingly.

2.8 Serum Urea Concentration

Serum urea levels were estimated by an assay Kit (Urea/BUN- Biosystems). The urea of the sample reacts with the kit's reagent components (urease, nitroprusside, salicylate, and NaClO), which forms a colored complex that was measured spectrophotometrically at 600 nm, and the concentrations were calculated accordingly.

2.9 Determination of oxidative stress biomarkers

2.9.1 lipid peroxidation

Lipid peroxidation (LPO) is a kidney injury indicator induced by reactive oxygen species (Kongkham et al., 2013). Malondialdehyde (MDA), as a marker for LPO, was determined by measuring thiobarbituric acid reactive substance (TBARS). Briefly, 0.5 ml of kidney tissue homogenates prepared were reacted with 2 ml of TBA reagent (0.375% TBA, 15% trichloroacetic acid, and 0.25 N HCl). Samples were boiled for 15 minutes, cooled, and centrifuged. The absorbance of the supernatants was measured spectrophotometrically at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (1.56 × 105 Statistical analysis M/cm), and expressed in µmol/100 g of tissue [18].

2.9.1 Superoxide dismutase (SOD) activity

The SOD activity was measured as follows: a certain amount of pyrogallol solution (60 mmol / 1 mmol HCl, 37 °C) was mixed with Tris-HCl buffer (pH 7.4,0.05 M, 37

°C) containing Na2EDTA(1 mmol). The total volume was adjusted by the buffer to 3000 µl, and the absorbance of the mixture without a sample (control) was measured spectrophotometrically at 325 nm every 30 s for 5 min at 37 °C. Secondly, the same amount of pyrogallol was added to the sample's homogenate, then repeated the previous steps. Enzyme activity, which corresponds to the amount of enzyme that inhibits auto-oxidation of pyrogallol by 50 % was calculated and expressed per mg of protein [18].

2.10 Histopathological examination

The morphological appearance of the kidney was examined macroscopically. The microscopic evaluation was performed by dehydrating the kidney tissue sections in ethanol, cleared in xylene, and then embedded in paraffin wax. Kidney sections were cut in 5 µm thickness and stained with hematoxylin and eosin dye. The histological features were examined by an unbiased pathologist blinded to the experimental design, performing a semi-quantitative analysis of the kidney sections slides using a light microscope. The examinations focused on renal glomerular injury and the severity of lesions was determined using scores on a scale of Grade 0: No injury, Grade 1: partial injury, and Grade 2: complete injury. This study also examined the renal tubules representing dilatation, vacuolation, and necrosis. Interstitial edema and medullary congestion were also assessed. The severity of these histological features was graded as follows: (Normal, less than 25%, 25- 50%, 50-75%, and more than 75%, and given degrees (0, 1, 2, 3, 4) respectively). At last, the presence of medullary vascular congestion was given Grade (1), and the absence was Grade (0) [19].

2.11 Statistical analysis

The statistical study was conducted by the Graph Pad Prism (9.4.1). The numerical data were expressed as (mean ± standard error of the mean SEM). Parametric data were evaluated using a one-way analysis of variance (ANOVA) test, followed by Tukey's multiple comparisons test. Categorical ordinal data was evaluated by the Mann–Whitney U test. The frequency of categorical binary data was evaluated by Fisher's exact test. The results were considered significant statically at $P<0.05$.

RESULTS

3.1 Total phenol and flavonoids contents

The total contents of phenols and flavonoids in the aqueous extract were shown in Table 1. The calibration curve of phenols and flavonoids were shown in Figure 1 and Figure 2 respectively.

Table1. Total phenol and total flavonoid contents of the cardamom aqueous extract

Total phenol (mg GAE/g)	Total flavonoids (mg QE/g)
23.44 ± 0.006	1.77 ± 0.0005
Data shown as average of triplicates ± SD, n = 3.GAE: Gallic acid equivalent, QE: Querecetin equivalent	

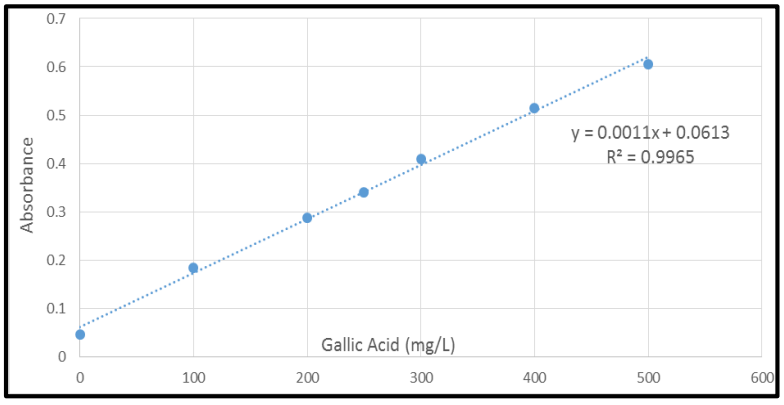

Figure 1. Calibration curve of phenol.

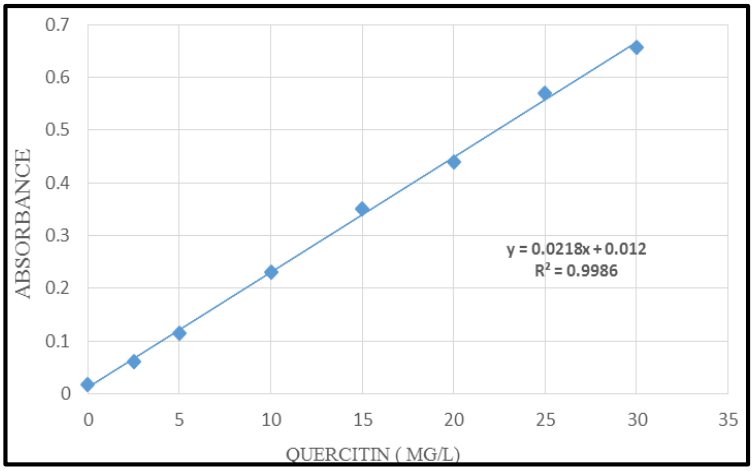

Figure 2. Calibration curve of Flavonoids

3.2 Body weight and Kidney weight/body weight ratio (%)

The body weight was reduced in the ASA group by (-14.6%) compared to the control and Card groups with statistical significances at (P<0.0001, P<0.01) respectively. While the Kidney weight/body weight ratio (%) was increased by (23.5%) compared to the control and Card groups with statistical significances at (P<0.01, P<0.05) respectively, (Table 2).

Table 2. Body weight, kidney weight and relative kidney weight of control and experimental rats.

Groups	Body weight(g)		Body weight change (%)	Absolute kidney weight (g)	Kidney weight/body weight ratio (%)
	Initial	Final			
Control	132.2±8.11	161.1±8.60	22.6±1.53	0.54±0.030	0.34±0.008
ASA	140.6±11.83	120.8±11.47	-14.6± 1.24****	0.51±0.021	0.42±0.021**
ASA + Card	129.7±9.99	118.2±8.68	-8.5±1.35##	0.41±0.030	0.35±0.023#

ASA: acetylsalicylic acid, Card: Cardamom extracts. All value represent mean ± SEM (n=8), Significance at P < 0.05. **Comparison to control group at P<0.01, **** Comparison to control group at P<0.0001.#Comparison to ASA group at P<0.05,##Comparison to ASA group at P<0.01.

3.3 Biochemical results

The levels of creatinine were significantly increased after aspirin administration compared with the control and Card group (P<0.0001.) (Figure 3). Also, the BUN was significantly increased in the ASA group (P<0.001.) (Figure 4). Cardamom extracts decreased the creatinine and BUN levels by (154.3% and 93.3%) respectively compared to the ASA group (Table 3).

Table 3. Results of creatinine, urea, lipid peroxides and SOD activity of control and experimental rats.

Groups	Serum parameters		Kidney tissue parameters	
	Creatinine (mg/dl)	Urea (mg/dl)	Lipid peroxides (µmol/ g tissue)	SOD activity
control	0.711±0.028	17.288±0.427	2.623±0.117	0.834±0.019
ASA	1.955±0.132****	39.513±2.126***	4.245±0.130****	0.563±0.038****
ASA + Card	0.787±0.033####	20.438±0.159####	2.962±0.030####	1.638±0.038####

ASA: acetylsalicylic acid, Card: Cardamom extracts. All value represent mean ± SEM (n=8), Significance at P < 0.05. ***Comparison to control group at P<0.001, **** Comparison to control group at P<0.0001. ####Comparison to ASA group at P<0.0001.

3.4 Oxidative stress biomarkers

Aspirin initiates an oxidative cascade within the kidney's tissue, which is reflected in elevating the LPO levels (38%) and declining SOD activity (49.7%) compared to the control group ($p < 0.0001$),(Table 3). Administration of cardamom aqueous extracts to the rats caused a significant reduction of the MDA levels in the kidney tissue ($p < 0.0001$) and a noticeable increase in SOD activity (65.6%) compared to the ASA group($p < 0.0001$) , (Figures 5 and 6).

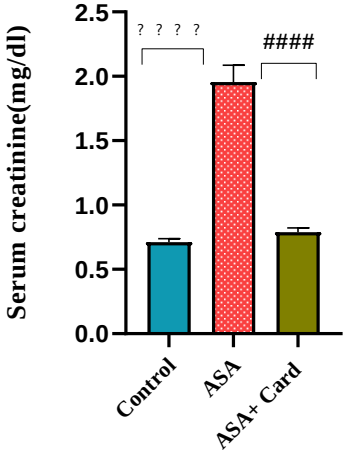

Figure 3. Serum creatinine levels in control and experimental rats groups. Values are expressed as mean±SEM

**** Comparison to normal control group at P<0.0001; ####Comparison to ASA group at P<0.0001, ASA: acetylsalicylic acid (600 mg/kg); Card: Cardamom (200 mg/kg)

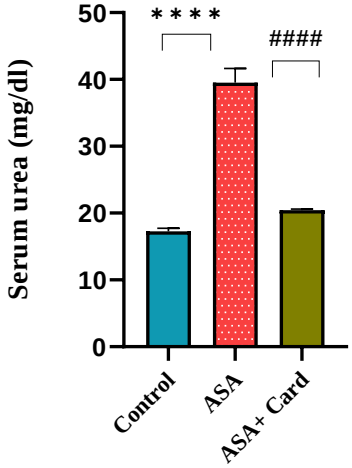

Figure 4. Serum urea levels in control and experimental rats groups. Values are expressed as mean ± SEM

**** Comparison to normal control group at P<0.0001; ####Comparison to ASA group at P<0.0001, ASA: acetylsalicylic acid (600 mg/kg); Card: Cardamom (200 mg/kg)

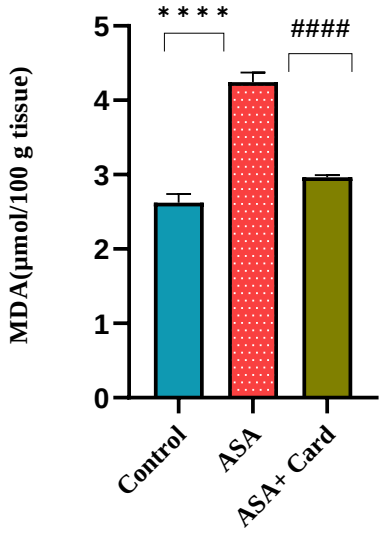

Figure 5. Lipid peroxidation levels in kidney tissue of control and experimental rats groups

Values are expressed as mean ± SEM. **** Comparison to normal control group at P<0.0001; #### Comparison to ASA group at P<0.0001, ASA: acetylsalicylic acid (600 mg/kg); Card: Cardamom (200 mg/kg).

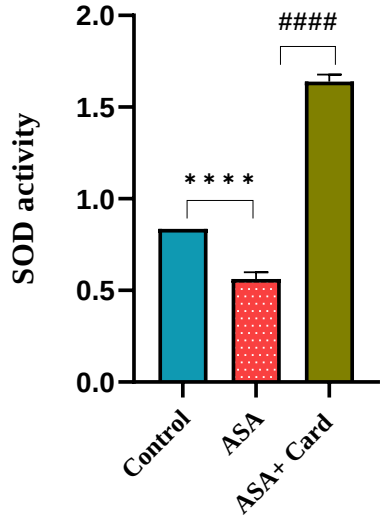

Figure 6. Superoxide dismutase (SOD) activity levels in kidney tissue of control and experimental rats groups.

Values are expressed as mean ± SEM. **** Comparison to normal control group at P<0.0001; #### Comparison to ASA group at P<0.0001, ASA: acetylsalicylic acid (600 mg/kg); Card: Cardamom (200 mg/kg)

3.5 Macroscopic examination

In the control group, the kidney had a normal macroscopic appearance with a smooth surface, while the sections showed the cortex and medulla (Figure 7:A). The kidney in the injured Group (ASA) had a bigger size and different macroscopic morphology when compared with the control group. The congestion and edema were observable, and the cortex color was pale (Figure 7: B). These macroscopic parameters and morphological changes were significantly reversed in the treatment group (Card), (Figure 7: C).

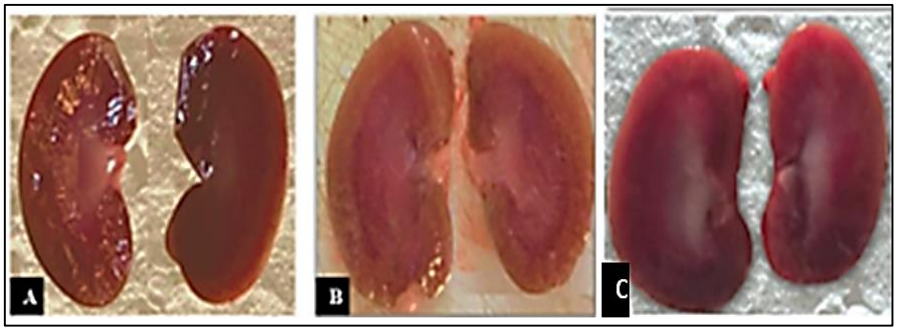

Figure 7. Macroscopic examination of the kidney sections in control and experimental rats groups. (A) Kidney with a normal macroscopic appearance in the control group. (B) Kidney is bigger and has noticeable congestion and edema with a pale cortex (ASA). (C) Morphological changes are improved in the cardamom treatment group

3.6 Microscopic Evaluation

The histopathological parameters were examined and scored (Tables: 4-5). The control group sections showed a normal glomerular appearance (Figure 6: A). Severe glomerular lesions were observed in the ASA group by the deformation of the extracellular matrix of glomerular ($p < 0.001$) compared to the control group (Figure 8: B)(Table 4). The tubule lesions showed in the ASA group, including dilatation, vacuolization, and necrosis, provided by swelling, fragmentation, and deformation of tubular epithelial cells resulting in high tubule injury scores compared to those of the control group ($p < 0.001$) (Figures 8:C, 6:D) and (Table 5). In addition, there was interstitial edema and medullary vascular congestion (Figures 8: B, 6: E), with significant static differences ($p < 0.0001$) (Table 5). While, the tubular vacuolization, necrosis, interstitial edema, and medullar congestion were significantly reduced in the cardamom group compared to the ASA group ($p < 0.01$, $p < 0.05$,$p < 0.01$, $p < 0.01$) respectively, (Figures 9:A, 7:B) and Table 4). There was a partial glomerular injury and tubule dilation with no significant statically difference in comparison with the ASA group ($p > 0.05$) and (Figure 9: C).

Table 4. Effects of aspirin and cardamom aqueous extracts on histopathological scores of a glomerular injury expressed as the frequency of injured rats in each group.

Groups	Grade 0	Grade 1	Grade 2	significance
		Histopathological Scores of glomerular injury		
Control	1 (12.5%)	7 (87.5%)	0	
ASA	0	0	8 (100%)	***
ASA +Card	2 (25%)	0	6 (75%)	ns

ASA: acetylsalicylic acid, Card: Cardamom extracts. Scores according to coverage of injury in glomerular, Grade 0: No injury. Grade 1: partial injury. Grade 2: complete injury. ns: non significance ($P > 0.05$), ***Comparison to control group at $P<0.001$.

Table 5. Effects of aspirin and cardamom aqueous extracts on histopathological scores of renal tubules (dilatation, vacuolization and necrosis), interstitial edema, and medullary congestion expressed as the frequency of injured rats in each group

Groups	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	significance
		Histopathological Scores of dilatation tubules				
Control	3 (37.5%)	3 (37.5%)	2 (25%)	0	0	
ASA	0	0	0	7 (87.5%)	1 (12.5%)	****
ASA + Card	0	0	4 (50%)	4 (50%)	0	ns
		Histopathological Scores of vacuolization tubules				
Control	3 (37.5%)	5 (62.5%)	0	0	0	
ASA	0	0	2 (25%)	5 (62.5%)	1 (12.5%)	****
ASA + Card	0	0	4 (50%)	4 (50%)	0	##
		Histopathological Scores of necrosis tubules				
Control	3 (37.5%)	5 (62.5%)	0	0	0	
ASA	0	0	0	5 (62.5%)	3 (37.5%)	****
ASA + Card	1 (12.5%)	3 (37.5%)	4 (50%)	0	0	#
		Histopathological Scores of interstitial edema				
Control	2 (25%)	6 (75%)	0	0	0	
ASA	0	0	2 (25%)	6 (75%)	0	****
ASA + Card	0	4 (50%)	4 (50%)	0	0	##
		Histopathological Scores of medullary congestion				
Control	7 (87.5%)	1 (12.5%)	0	0	0	
ASA	0	1 (12.5%)	3 (37.5%)	3 (37.5%)	1 (12.5%)	****
ASA + Card	0	0	4 (50%)	4 (50%)	0	##

ASA: acetylsalicylic acid, Card: Cardamom extracts. Scores according to percent of injury in tubular epithelium, Grade 0: normal, Grade 1(< 25%), Grade 2 (25–50%), Grade 3 (50–75%), Grade 4 (>75%). ns: non significance ($P > 0.05$), ****Comparison to control group at $P<0.0001$, ##Comparison to ASA group at $P<0.01$ #Comparison to ASA group at $P < 0.05$.

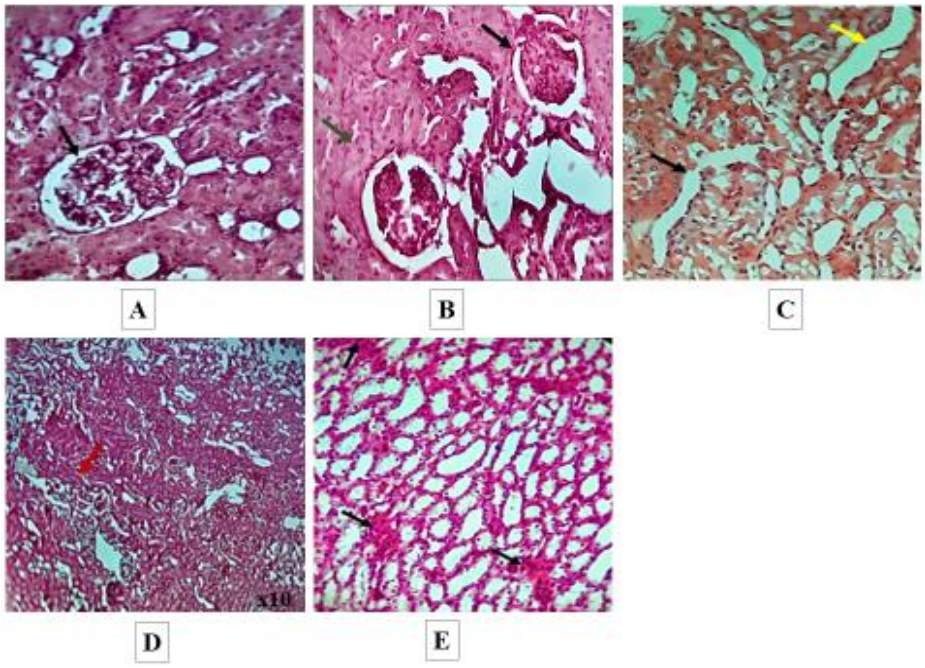

Figure 8. Histopathological changes in aspirin-induced nephropathy (600mg/kg) dose in rats (Hematoxylin and eosin x40). (A Control group): normal glomerulus, (B, C, D and E: Aspirin group). (B) Complete glomerular injury (black arrow) and interstitial edema (green arrow). (C) Abnormal dilatation (yellow arrow) and vacuolization (black arrow) of the renal tubules. (D) Total necrosis and deformation (red arrow) x10. (E) Medullary vascular congestion (black arrow).

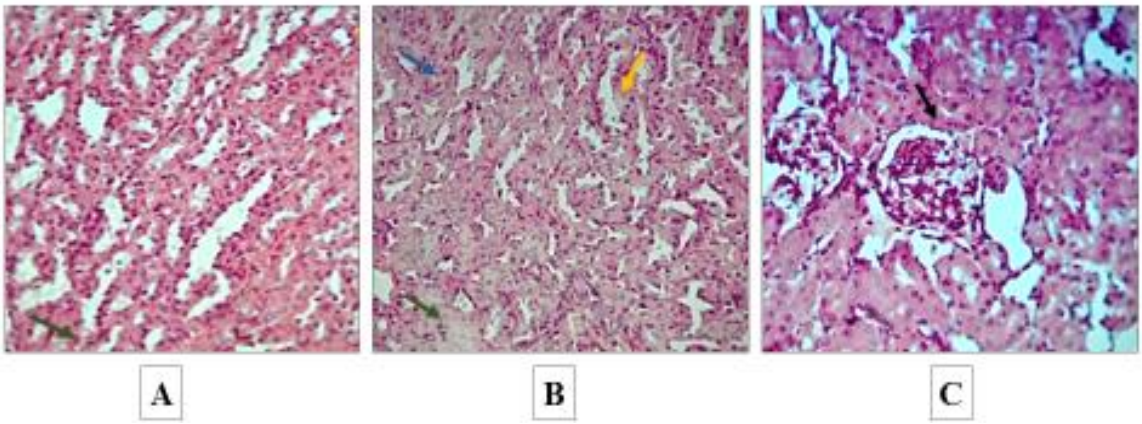

Figure 9. Effect of Cardamom aqueous extract (200 mg/Kg) on histopathological changes in (600mg/kg) dose aspirin-induced nephropathy in rats. (A, B, and C: Treatment group Card: hematoxylin and eosin x40). In (A) mild interstitial edema (green arrow) and less congestion, (B) mild tubular dilatation (yellow arrow) and vacuolization (blue arrow) in tubules, (C) partial injury to the renal glomerular

4. DISCUSSION

Due to the importance of limiting Aspirin-induced nephropathy, this study aims to investigate the effect of cardamom on controlling this damage. As the first trial was studied the aqueous cardamom extracts therapeutic efficacy on aspirin nephropathy based on biochemical and histological data.

The findings of this study revealed that aspirin kidney damage was in accord with the changes in the renal function tests and oxidative stress biomarkers. In this concern, alteration of the function of renal biochemical parameters, oxidative stress biomarkers, and histopathological studies confirmed the therapeutic effect of cardamom aqueous extract. Taking into consideration that cardamom is one of the most effective plants that scavenge free radicals and suppress their oxidative damaging effects [20]. The antioxidant activity of cardamom was attributed mainly to its high contents of phenolic and flavonoids. Thus, in our study, the total phenols and flavonoid contents in aqueous cardamom extract (Table 1). Investigation of the cardamom pharmacological effects representing in phenolic and flavonoid compounds has been suggested as possible effecting factors in previous studies [21–23].

Aspirin could induce mitochondrial dysfunction by acetylate proteins altering their morphology and leading to uncontrolled biomolecule oxidation, which increases ROS [24],and elevates plasma pro-inflammatory cytokines (IL-6, IL-8, and TNF-α), which promote oxidative stress and increases the possibility of the development of kidney diseases [25]. In addition, aspirin inhibits the mitochondrial electron transport chains and causes irreversible oxidative damage [26].The induced kidney injury and inflammation caused by aspirin administration increased kidney weight. However, because of the decreased appetite and reduction in food intake, the body-weight ratio declined compared to the control group. Therefore, the kidneys are oversensitive to reactive oxygen species (ROS) harm. Thus, increased kidney weight might be related to inflammation, edema, and oxidative stress following aspirin (600 mg/kg). These results correlated with the previous studies of [2] and [26]. In this context, cardamom elevated the body weight ratio and decreased the relative weight of the kidney in the cardamom treatment group compared to the aspirin group, which attributed mainly to flavonoids mechanism in

suppressing the NF-κB inflammation and preventing the expression of proinflammatory cytokines (IL-6, IL-1β, TNFα) [27]. That partly disagrees with the study of Aboelnaga with cardamom reduced body weight, while it is compatible with our results in decreasing relative kidney weight [28].

Creatinine and urea are still the most usable and significant indicators in renal function estimation [29]. Creatinine and urea levels were significantly elevated in the aspirin group compared to the control group, in agreement with the study of Ogunjemite et al. In a way, aspirin inhibits the synthesis of PGE2 and PGI2, causing a decrease in renal blood flow that may lead to impaired renal function, reflected by increased creatinine and urea levels [30]. The cardamom reversed the harmful effect of aspirin by improving kidney functions represented in creatinine and urea, which were reported earlier in other models of renal impairment induced by gentamicin by [13] and in a previous study by [20] in doxorubicin nephrotoxicity.

Renal ischemia caused by aspirin may stimulate the production of ROS [24], which initiates lipid peroxidation observed by elevating MDA levels and altering the endogenous antioxidant SOD functions and concentrations too [31]. Accordingly, to these cellular variations that confirmed renal tissue injury, aspirin caused a significant increase in MDA levels and a decrease in SOD activity in the current study, compatible with the results reported by [32]. On the other hand, there was a significant increase in SOD levels and a decrease in MDA levels in the cardamom treatment group. Due to its richness in potent phenolic and flavonoid antioxidant compounds suppressing lipid peroxidation and neutralizing the free radicals activity, in agreement with a previous study by [33] that detected the protective role of cardamom against diethylnitrosamine-induced oxidative stress in the kidney. The histopathological evaluation supported biochemical findings and indicated that cardamom has an antioxidant effect on the renal damage induced by aspirin. Whereas in our study, aspirin-induced renal damage was associated with tubular injuries (dilatation, vacuolization, and necrosis) and glomerular degeneration. These results are consistent with the results of previous studies [2,34].

The current study indicated that cardamom moderates tubular injuries and reduces congestion compared to the aspirin group. According to Khattab et al previous study, cardamom caused a histological improvement in renal tubule vacuolization, which could be attributed to its antioxidant and free radical scavenging properties [35].

CONCLUSIONS

In summary, the current study demonstrated the therapeutic effect of cardamom against aspirin-induced nephropathy. Cardamom has reduced kidney injury by improving the histological, biochemical, and kidney oxidative stress parameters. The antioxidant bioactivity of cardamom is explained by its high content of phenols and flavonoids.

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تقييم فعاليّة خُلاصة حب الهال في علاجِ الاعتِلالِ الكُلويّ المُحدَث بالأَسبرين عِندَ جرذانِ التجربة

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ملخص

يعدّ الاعتلال الكلوي بمسكنات الألم أحد الاختلاطات الناتجة عن الاستخدام المفرط والمزمن للمسكنات، وخاصةً التي تصرف بدون وصفة طبية مثل الأسبرين. تهدف هذه الدارسة إلى تحرّي التأثير العلاجيّ لخلاصة الهال على الاعتلال الكلويّ المُحدث بالأسبرين عند جرذان التجربة. وُزعَ 24 جرذاً إناثاً من فصيلة Albino Wistar على ثلاث مجموعات (8 حيوانات/مجموعة): (Control) المجموعة الشاهدة الطبيعية، (ASA) الأسبرين (600 ملغ/كغ/اليوم مدّة 4 أيام)، (ASA + Card 100): الأسبرين + خلاصة الهال (100 ملغ/كغ/اليوم مدّة 7 أيام)، و (ASA+ Card 200): الأسبرين + خلاصة الهال (200 ملغ/كغ/اليوم مدّة 7 أيام. حُضِّرَت الخلاصة المائية للهال. حُسب محتواها من الفينولات والفلافونوئيدات. حُسبت نسبة وزن الكلية/ وزن الجسم، وقيّست مستويات البولة والكرياتينين المصليين. قُيّمتْ مستويات فوق أكاسيد الشحوم بقياس تراكيز Malondialdehyde (MDA) وفعالية أنزيم Superoxide dismutase (SOD) في نسيج الكلية، بالإضافة للفحوص النسيجية. قُيّمت البيانات المعلمية باستخدام اختبار التباين الأحادي (ANOVA) One-way analysis of variance متبوعاً باختبار Tukey's. والبيانات اللامعلمية قُيّمت باختبار Mann–Whitney متبوعاً باختبار Ficher، عُدَّت النتائج جوهرية عندP<0.05. قُدِّرَ محتوى الفينولات 23.4 مغ مكافئ حمض الغاليك/غ خلاصة جافة، والفلافونوئيدات 1.77 مغ مكافئ كيرستين/غ خلاصة جافة. لوحظ ارتفاعاً معتداً به إحصائياً في نسبة وزن الكلية/ وزن الجسم، وفي البولة والكرياتينين المصليين، وتراكيز MDA، في حين انخفضت فعالية SOD وذلك بمقارنة مجموعة الأسبرين مع المجموعة الشاهدة الطبيعية. كما أظهر الفحص النسيجي أذيّاتٍ نبيبية وكبيبية معتدّاً بها إحصائياً. لوحظ تحسّناً جوهرياً في معظم المعالم المصليّة والنسيجيّة لدى مجوعة العلاج Card 200 لدى مقارنتها مع مجموعة الأسبرين. أظهرت خلاصة نبات الهال (200 مغ/كغ) فعالية علاجيّة تِجاه الاعتلال الكلويّ المُحدث من خلال تحسن وظائف الكلية، والمعالم الأنزيمية والنسيجية، وذلك بفضل فعاليته المضادة لحالة الإجهاد التأكسدي المُحدث بالأسبرين.

الكلمات الدالة: الكلية، الاعتلال الكلوي، مضادات الالتهاب اللاستيروئيدية، الأسبرين، خلاصة الهال، الإجهاد التأكسدي.

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Exploring Anti-inflammatory Targets of Flavonoids through Integrated Molecular Docking and Network Pharmacology

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ABSTRACT

Inflammation is a complex physiological response associated with numerous diseases. Flavonoids, a class of natural compounds widely distributed in plants, have demonstrated promising anti-inflammatory properties. However, their comprehensive mechanisms of action and potential molecular targets remain indefinable. In the present study, we employed a network pharmacology approach combined with molecular docking to investigate the anti-inflammatory effects of some flavonoids. Initially, we collected and curated a comprehensive database such as *ADMET* parameters and targets from Swiss *ADME*, *ADMET* 2.O and *Swiss* target predication. We further constructed a protein-protein interaction network to identify key proteins involved in inflammation by using string database. Subsequently, we integrated the flavonoid dataset with the protein network to establish potential flavonoid-protein interactions by using *Cytosacpe* vina. The *GO* and *KEGG* enrichment analysis were done with the help of David database. Molecular docking was accomplished through Autodock Vina, and assessed the binding affinity of selected flavonoids towards the identified target proteins. The docking analysis provided insights into the specific interactions between flavonoids and target proteins, elucidating the potential mechanisms underlying their anti-inflammatory effects. The bioactive components ferulic acid, quercetin, rutin and hesperidin modulates many molecular and cellular processes and then exerts anti-inflammatory effects. From the analysis the key targets were participated in inflammatory bowel disease, IL 17 signaling pathway, TNF signaling pathway, cytokine-mediated signaling pathway, rheumatoid arthritis, lipopolysaccharides etc. Further molecular docking studies also revealed that binding affinity of selected flavonoids were higher than that of diclofenac.

Keywords: Anti-inflammatory; Ferulic acid; Hesperidin; Molecular docking; Network Pharmacology; Quercetin; Rutin.

INTRODUCTION

Globally, it is acknowledged that inflammatory illnesses constitute a substantial contributor to morbidity in humans [1]. Inflammation is a fundamental biological response of the immune system to tissue injury, infection, or other harmful stimuli [2]. It is a complex process involving various immune and non-immune cells, chemicals, and molecular signaling pathways that work together to protect the body and initiate the healing process [3]. There are various types of inflammation, while acute inflammation is an essential part of the body's defense mechanism, it is characterized by redness, heat, swelling, discomfort, and functional loss [1] and it is a vital part of the immune response and plays a crucial role in initiating the healing process. Whereas chronic inflammation can lead to tissue damage and contribute to the development of

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numerous diseases [4] such as multiple sclerosis, psoriasis, inflammatory bowel syndrome, autoimmune disorder (rheumatoid arthritis), cardiovascular diseases, atherosclerosis and metabolic conditions [5, 6].

Inflammation is orchestrated by a network of signaling molecules including various immune cell. Cytokines have both inflammatory and anti-inflammatory response. Thus, IL-6, tumour necrosis factor (TNF), and IL-18 are pro-inflammatory cytokines which initiate and promote inflammatory processes [7]. Whereas IL-10, inflammatory receptor agonist (IRA), and transforming growth factor (TGF) are examples of anti-inflammatory cytokines that adversely affect these processes (Figure 1). Subsequently, prostaglandins, neutrophils and macrophages, chemokines are also act as chemical messengers to regulate the immune response. These molecules attract immune cells to the site of inflammation, promote their activation, and coordinate the overall immune response [8].

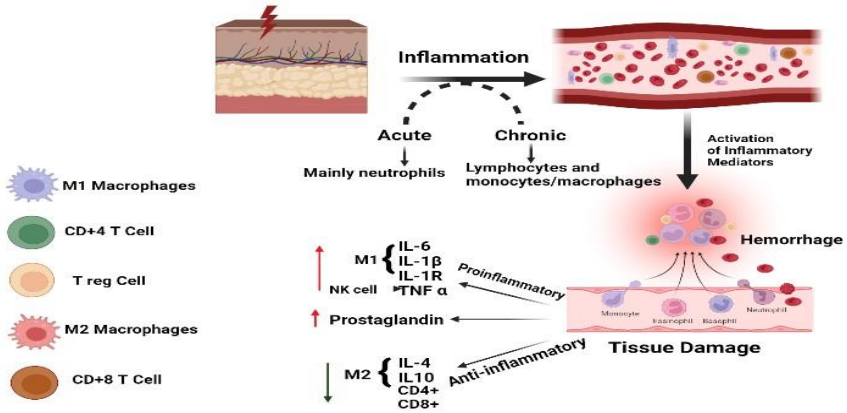


Figure 1. Various inflammatory mediators which regulate inflammatory responses

Currently for the management of the inflammation widely used synthetic drug out of 5% prescribe medicine NASIDS (Non-steroidal anti-inflammatory drugs) [9] like Diclofenac, ibuprofen [10] which inhibits cyclooxygenase (COX) and prostaglandin, its control the release of interleukin (IL) 6 and IL-10 by triggering cAMP and it inhibits many inflammatory mediators [11]. There are many adverse drug reactions of anti-inflammatory drugs. Recently, more emphasis on the examination of medicinal plants to discover new component and validate their traditional uses for the control of inflammatory diseases and for the development of safer and efficient anti-inflammatory agents [12].

In recent years research on the anti-inflammatory properties of the various flavonoids has increased. Flavonoids are a class of organic compound with multiple phenolic structures [13]. The major source of flavonoids are fruits and vegetables. Several studies have demonstrated that flavonoids have many pharmacological properties like anti-inflammatory, antiviral, antioxidant, antiallergenic antineoplastic activities [14, 15]. Flavonoids such as ferulic acid, quercetin, rutin, hesperidin has properties to counteract inflammatory cytokines, which modifying inflammation-related pathways, and controlling inflammatory diseases [16, 17].

Network pharmacology has already emerged as a valuable and indispensable tool for the in-depth study of traditional Indian medicine, contributing to its integration with modern medicine and promoting the development of innovative and effective therapies. network pharmacology has developed into a frequently used analytic technique, promoting the development of the areas of systems biology

and pharmacology [18, 19]. It helps to discovered new mechanism of action towards multiple target and diseases with the help of protein and gene interaction with bioactive molecules.

Molecular docking is most popular bioinformatics modelling in rational drug discovery and structure-based drug design. molecular docking is a computational tool which help to visualizes virtual interaction between protein-ligand molecules [20]. The docking technique's outputs may be used to estimate the binding energy, free energy, and stability of complexes [21].

MATERIALS AND METHODS

1. Selection of bioactive compounds

Flavonoids are the secondary metabolites responsible for an anti-inflammatory potential. Therefore, ferulic acid, quercetin, rutin and hesperidin were selected for the study as these flavonoids are most abundant in maximum of the plant's families.

2. ADME-T Analysis of Bioactive Compounds

In the context of network pharmacology, ADME-T (absorption, distribution, metabolism, excretion, and toxicity) study is a crucial component for the selection of bioactive compound. The Swiss ADME (http://www.swissadme.ch/) and ADMET 2.0 (https://admetmesh.scbdd.com/) online web tool were used to obtain ADME- T properties of compounds [22, 23].

3. Screening of Active Phytoconstituents for Target Predication

The phytoconstituents were screened for its possible target using Swiss target predication (http://www.swisstargetprediction.ch/) and uniport Id was collected from uniport database (https://www.uniprot.org) [24, 25].

3.1 Establishment of a Database of Inflammatory Gene Target

Genecard database (https://www.genecards.org/) online tool was used for the searching of the target for the inflammation [26]. Multiple targets were identified and duplicate targets were removed and processed for Venn diagram construction.

3.2 Construction of Venn Diagram

Venn diagram web tool (https://bioinformatics.psb.ugent.be/webtools/Venn/) generally give information about common targets involved in the drug and disease, based on which one can predict most common targets associated with disease and drug [27].

3.3 Construction of Protein-Protein Interaction

To clear the visualization and understand the molecular mechanism of the targets, it is important to study the Protein-Protein interaction (PPI) of the target genes. The target genes of the corresponding components will be subjected to STRING v12.0 (https://string-db.org/) to visualize and construct the PPI network [28]. The excel sheet of common target gene was upload into string data base where "*Homo sapiens*" species was selected, and medium confidence and "FDR stringency" was set at "medium 5%" in "required score" and construct PPI network and further screened for core genes [29].

3.4 GO Function and KEGG Enrichment Analysis

Gene ontology (GO) enrichment analysis was carried out to analyze the target proteins and KEGG analysis was carried to determine the signaling pathway [30]. David database (https://david.ncifcrf.gov/) was utilized to set the analysis condition "official gene symbol" "*Homo sapiens*" for the KEGG pathway enrichment study and GO function analysis of the inflammatory targets [31]. The pathway was examined in biological process (BP), responsible for inflammatory effect and standard was set as $P< 0.05$. The top 10 genes with relatively minimum P value were screened out in cellular component (CC) and molecular function (MF) respectively. BP, CC, MF data was analyzed and mapped with the help of bioinformatics analysis and visualization of cloud platform online tools (https://www.bioinformatics.com.cn/en). From the analysis of KEGG pathway, "enrichment bar diagram" was fashioned to visualize inflammatory conditions [32].

4. Molecular Docking

Molecular docking is a computational technique used to predict the binding orientation and affinity of a small molecule (ligand) to a target protein or receptor [33]. It plays a crucial role in drug discovery and design by aiding in the identification of potential lead compounds [34]. Using PDB database (https://www.rcsb.org/) we retrieve molecular structure file of the target protein. Autodock vina (version 1.5.7) software used for molecular docking. Hydrogen atoms was added to assign appropriate charges to the protein structure [35]. PyMOL 2.3.0 software was used to download original ligands of target proteins and Pubchem (https://pubchem.ncbi.nlm.nih.gov/) was used to download computational structure files of ligand. Sequentially, Target protein prepared with Autodock vina, target protein rang and location were set for inhibitor after applying grid box to standard compound [36]. The flow chart of whole process of study was depicted in Figure 2.

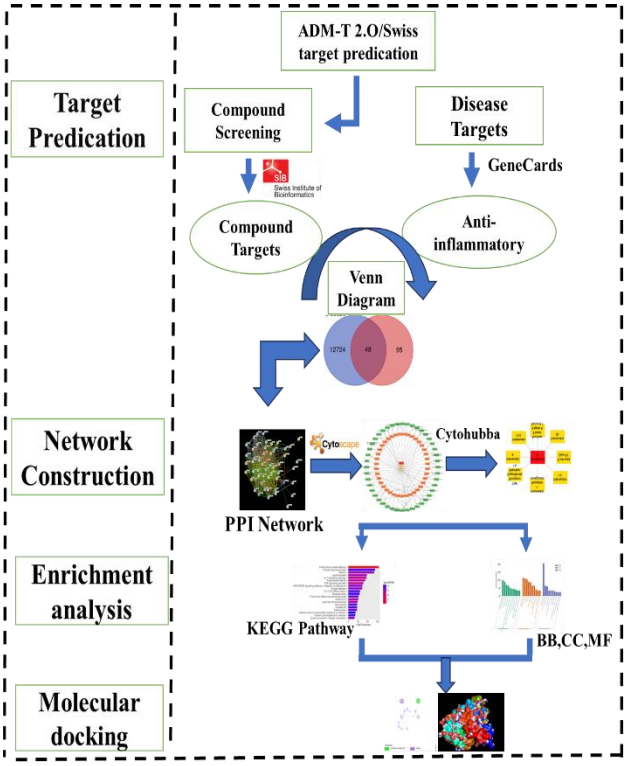


Figure 2. Flow chart of study to explore a potential target of flavonoids for anti-inflammatory activity

RESULTS

Physicochemical parameters and ADMET Analysis

The physicochemical properties and pharmacokinetics parameters of bioactive compounds was examined and discussed in Table 1. Lipophilicity, absorption, distribution, Metabolism, excretion, toxicity of bioactive compound was classified into eight different groups. The oral bioavailability chart of the bioactive compound is mentioned in Figure 3. HIA and BBB penetration are essential parameters that are evaluated during the drug development process. The topological polar surface area (TPSA) is commonly used metric for optimizing a drugs ability to permeate cell membrane. TPSA of CNS drugs usually lower than the non-CNS drugs. Drugs with a lower TPSA (generally below 90 A^2) tend to permeate the BBB more easily, allowing them to reach the brain and exerts

their therapeutic effects. Ferulic acid more easily cross the BBB than other flavonoids as it shown lower TPSA (66.76 Å²). Similarly, the BBB penetration ability of flavonoids is in the range of ferulic acid > quercetin > hesperidin > rutin. These parameters help in assessing the absorption characteristics of a drug and its potential effects on the central nervous system.

Table 1: ADMET information of Ferulic acid, Quercetin, Rutin, Hesperidin

Properties	Parameters	Ferulic acid	Quercetin	Rutin	Hesperidin
Physiochemical	MW[a](g/mol)	194.18 g/mol	302.24 g/ml	610.52 g/mol	610.19
	Rotatable bonds	3	1	6	7
	Fraction Csp3	0.10	0.00	0.44	0.536
	Molar Refractivity	51.63	78.03	141.38	0.54
	TPSA	66.76 Å²	131.36 Å²	269.43 Å²	234.29 Å²
Lipophilicity Log Po/w	iLOGP	1.62	1.63	1.58	2.60
	XLOGP3	1.51	1.54	-0.33	-0.14
	WLOGP	1.39	1.99	-1.69	-1.48
	MLOGP	1.00	-0.56	-3.89	-3.04
	SILICOS-IT	1.26	1.54	-2.11	-1.55
	Consensus Log Po/w	1.36	1.23	-1.29	-0.72
Absorption	GI absorption	High	High	Low	Low
	skin permeation	-6.41 cm/s	-7.05 cm/s	-10.26 cm/s	-10.12 cm/s
Distribution	PPB	89.754%	95.496%	83.811%	77.64%
	VD	0.339	0.579	0.754	0.395
	BBB Penetration	Yes	Yes	No	Yes
Metabolism	CYP1A2 inhibitor	No	Yes	No	No
	CYP2C19 inhibitor	No	No	No	No
	CYP2C9 inhibitor	No	No	No	No
	CYP2D6 inhibitor	No	Yes	No	No
	CYP3A4 inhibitor	No	Yes	No	No
Execretion	Rate of clearance	7.480	8.284	1.489	1.489
Toxicity	hERG Blockers	0-0.1	0-0.1	0-0.1	0-0.1
	Hepatotoxicity	0.3-0.5	0-0.1	0-0.1	0-0.1
	AMES Toxicity	0.1-0.3	0.5-0.7	0.3-0.5	0.3-0.5
	FDAMDD	0-0.1	0.3-0.5	0-0.1	0-0.1
	Skin Sensitization	0.9-1.0	0.9-1.0	0-0.1	0-0.1
	Carcinogenicity	0.3-0.5	0-0.1	0.7-0.9	0.7-0.9
	Eye Irritation	0.9-1.0	0.9-1.0	0-0.1	0-0.1

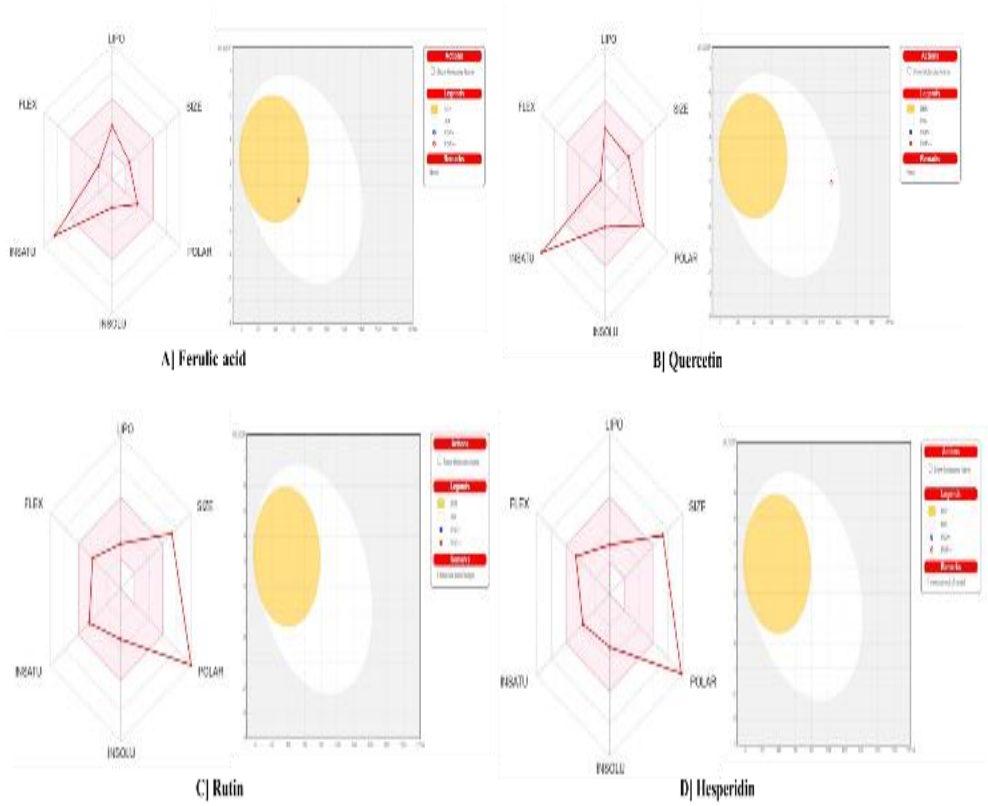


Figure 3. Bioavailability chart for bioactive compounds. The red line indicates oral bioavailability properties and the pink region represents the physicochemical space for oral bioavailability

Acquisition of Target for Inflammation/ Construction of Venn Diagram

There are about 12727 targets for inflammation as per Genecard database. While 97 diseases are associate with ferulic acid, 96 with quercetin and rutin, 95 with hesperidin as per Swiss Target Predication. Out of this 45 common target for Ferulic acid, 55 for Quercetin, 46 for Rutin, and 48 for Hesperidin was observed by Venn diagram.

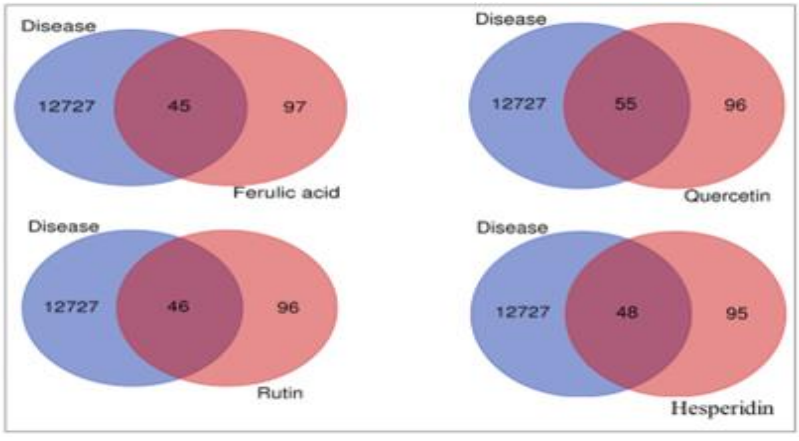


Figure 4. Common target analysis of phytoconstituents with disease targets. The blue color denotes the disease targets and red colour represents the drug targets and the intersection points of circle represents the common targets

GO and KEGG Enrichment analysis

The target genes of the common intersection were examined using the Database for Annotation, Visualization, and Integrated Discovery (David database's), where we performed Gene ontology (GO) enrichment analysis (Figure 5) and Kyoto Encyclopedia of Gene and Genomes (KEGG) to gather the corresponding biological functions and processes (Figure 6). GO enrichment analysis was mainly involved in GO biological process (BP), GO cellular component (CC), and the GO molecular function (MF) these terms were plotted using Bioinformatics tool for the analysis and visualization of data.

Through analysis it was found that the BP of anti-inflammatory effect of ferulic acid, quercetin, rutin and hesperidin mainly focused on GO:1900407~ regulation of cellular response to oxidative stress, GO:0019221~cytokine-mediated signaling pathway, GO:0006954~inflammatory response, GO:0045087~innate immune response etc. CC focused on GO:0005886~plasma membrane, GO:0005615~extracellular space, GO:0005576~extracellular region etc, MF is mainly supplemented in GO:0005515~protein binding, GO:0042803~identical protein binding etc. Analysis of BP, CC and MF data are depicted in F**igure 5**. From the BP analysis it was postulate that anti-inflammatory response of hesperidin was higher when compared with ferulic acid, quercetin and rutin. In CC analysis involvement of extracellular space was observed with all phytoconstituents whereas; in MF analysis most of the phytoconstituents depicted high protein binding when compared with other GO terms associated with MF.

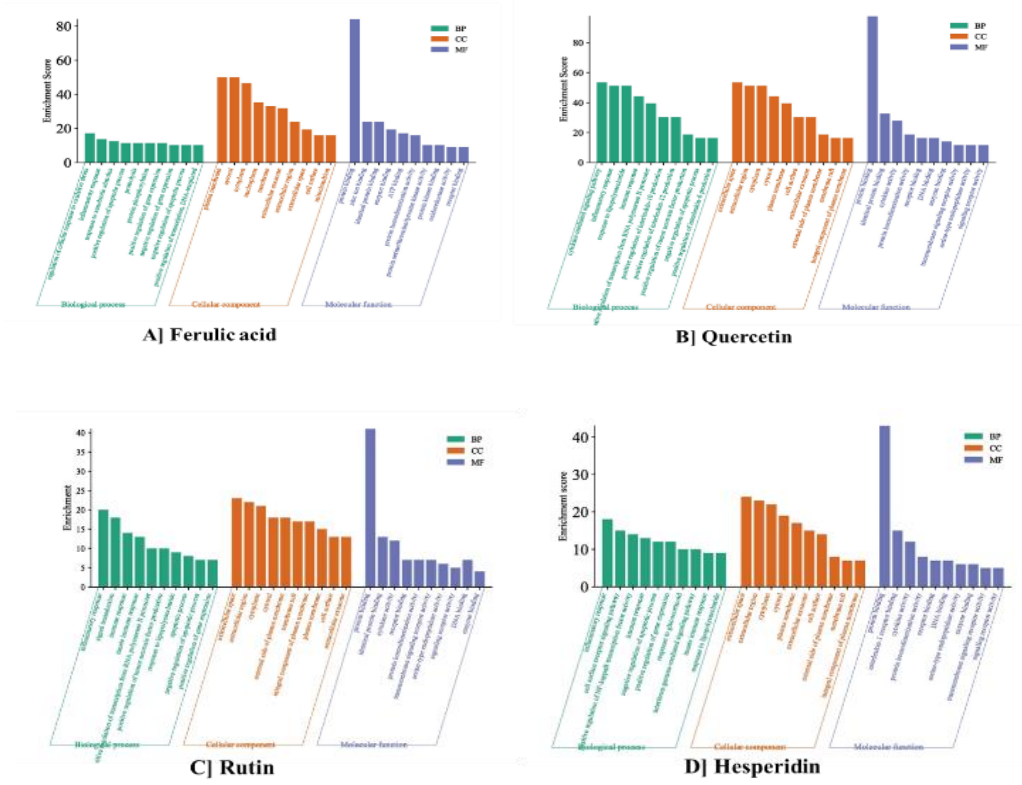


Figure 5. GO enrichment analysis of common targets of the Ferulic acid, Quercetin, Rutin and Hesperidin against inflammation

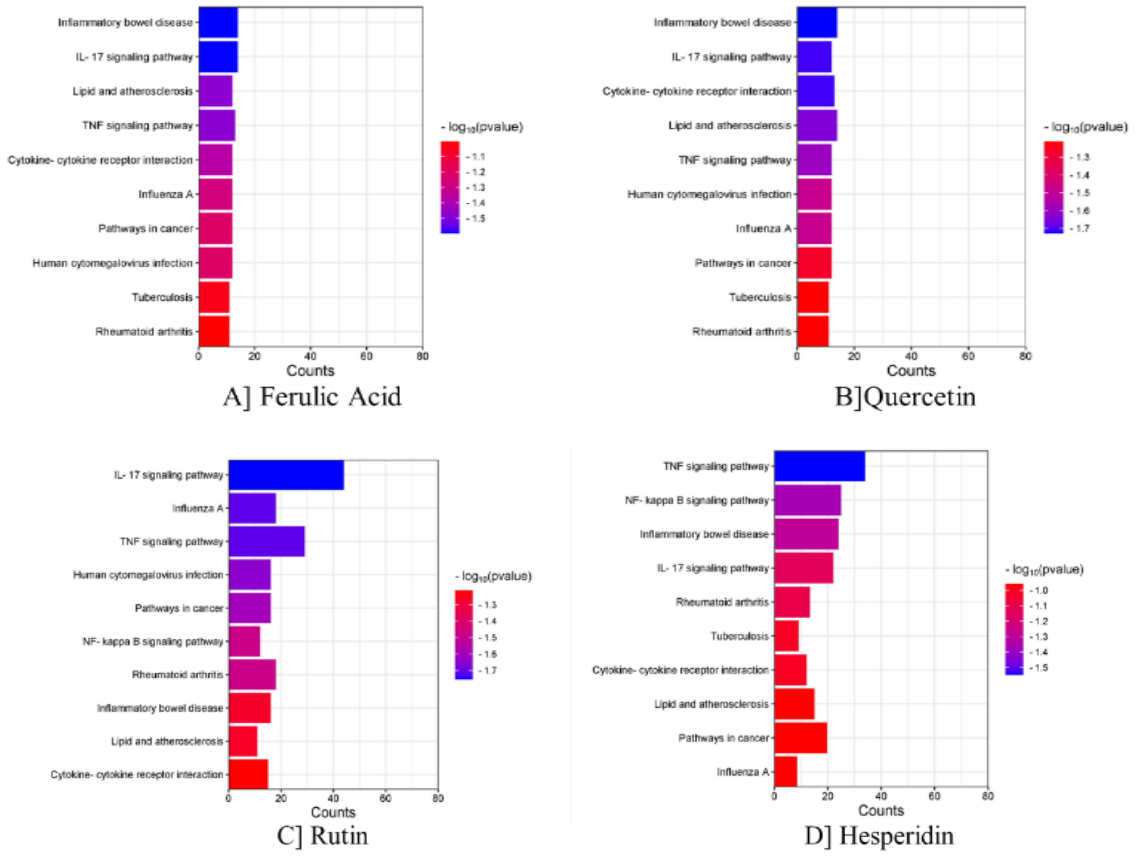


Figure 6. KEGG function analysis

David database was used to analyze the function of KEGG pathway. In figure 6 (A, B, C & D) from the data it was observed that involvement of various pathways those were associated with inflammation among them 74 pathways for ferulic acid; 82 pathways for quercetin; 70 pathways for rutin and 73 pathways for hesperidin. From these data we selected top 10 pathways those were highly associated with inflammation for bar plot analysis. In present analysis P<0.05 were selected as a level of significance; and smaller the P value higher the anti-inflammatory effect.

PPI Network Construction and Analysis

PPI network used to identify the relationship between common targets of bioactive compound with help of string database. All common targets of bioactive compounds showed protein-protein interaction. We got 48 nodes and 568 edges for ferulic acid, 53 nodes and 635 edges for quercetin, 42 nodes and 480 edges for rutin and 43 nodes and 448 edges for hesperidin. PPI network showed the P value < 1.0 e-16 which showed better anti-inflammatory response (Figure 7). Illustration and visualization of PPI network was done by using Cytosacpe whereas; simplification and analysis of network was done by Cytohubba. The top 10 key genes of ferulic acid, quercetin, rutin and hesperidin was shown visually (Figure 8) according to the network, as follows: Ferulic acid (IL17A, IL4, IL13, IFNG, IL1A, IL1RN, NFKB1, IL18, PRTN3, TNFRSF1A) Quercetin (IL1RN, IFNG, MAPK14, PTK2B, GLO1, PDE5A, TLR4, IL10, IRF5, IL6) Rutin (IL17A, IL10, FOXP3, MPO, MMP9, FAS, ICAM1, TNFAIP3, ILRUN, RIPK1) and Hesperidin (NFKB1, FOXP3, IL10, IL17A, IL6, IFNG, IL1RN, TLR2, TLR4, PTGS2).

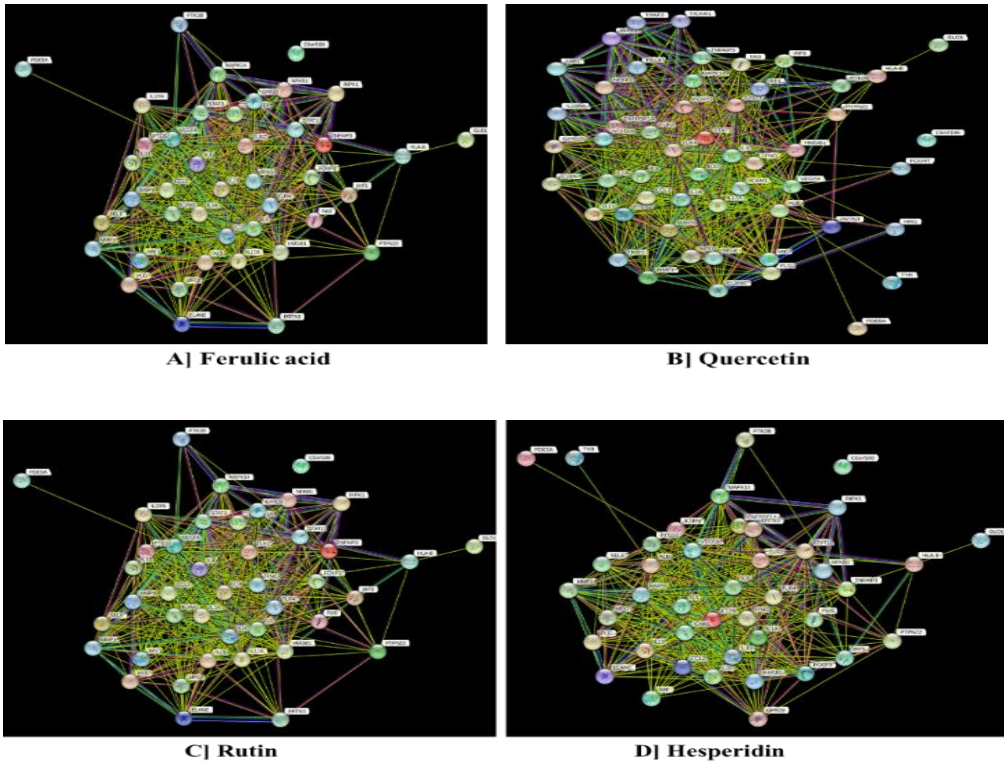
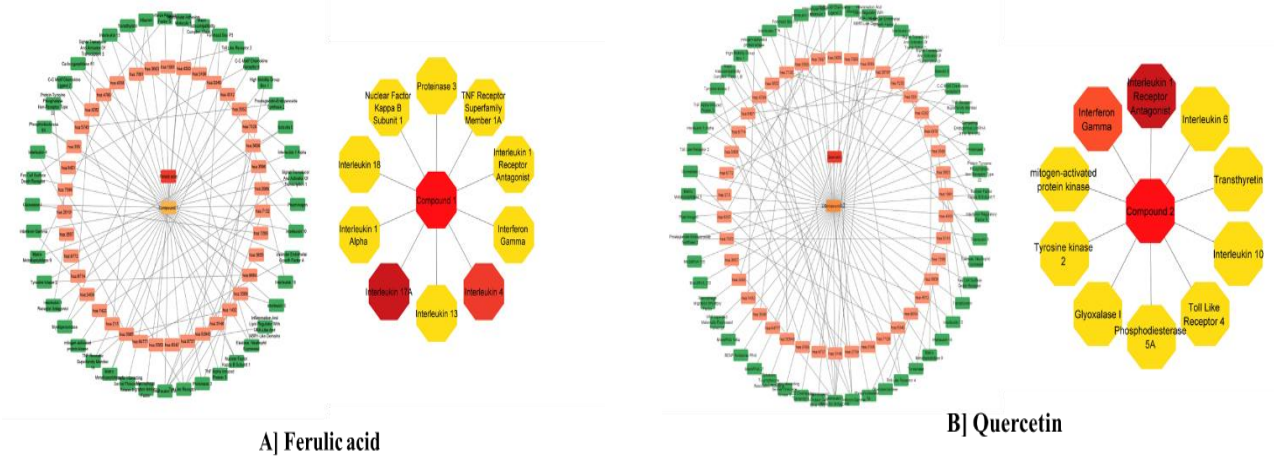


Figure 7. PPI network showing association of nodes and edges responsible for inflammation; these targets were used for computational modelling.



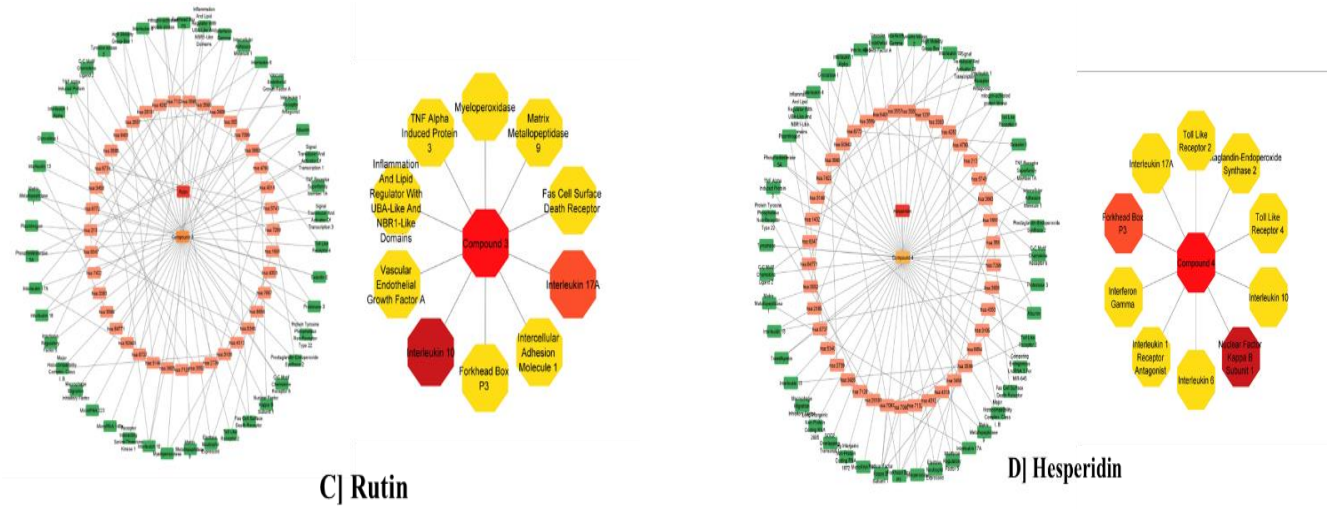


Figure 8. Compound-target pathway network of ferulic acid, quercetin, rutin and hesperidin are represented in fig A, B, C and D and the involvement of degree of interaction between top 10 targets with colors; yellow < orange < brown.

Molecular Docking

After applying Hubba to top 10 targets we identified two proteins from each active compound for molecular docking. On the basis of these, we selected IL 17A and IL4 as target for molecular docking of ferulic acid, IL1RN and IFNG for quercetin, IL17A and IL10 for rutin, NFKB1 and FOXP3 for hesperidin. These targets were selected because they show highest degree of interaction between respective drug and target (Figure 8).

The binding energy in molecular docking refers to the strength of the interaction between a ligand and a receptor. It also quantifies the stability of the complex formed between the ligand and the receptor. The binding energy is typically expressed in terms of a negative value, indicating the favorable nature of the interaction. If negative binding energy is larger than ≤ - 0.5 kcal/mol then stronger binding affinity between the ligand and the receptor. Then Protein-ligand interaction was performed for the selected bioactive compounds. Where, molecular docking results of all four-compound shown significant binding at the pocket site of the protein and Diclofenac was considered as standard control (Table 2).

Quercetin shown less binding free energy with IL1RN (-8.8) and -6.1 against the IFNG receptor than the diclofenac (-7.8 and -5.5 respectively) which indicate significant binding of quercetin and more potent effect than the standard drug (Table 2). Similarly, molecular docking score of rutin [against IL17A (-7.1) & IL10 (-7.7)] and hesperidin [against NFKB1 (-9.7) & FOXP3 (-9.4)] was found to be less than the standard drug diclofenac as displayed in Table 2. It indicates that all the selected flavonoids revealed potent anti-inflammatory potential against various targets of inflammation. Ferulic acid revealed anti-inflammatory potential by binding with IL17A and IL4 with docking score of -5.7 and -5.2 respectively as shown in Figure 9. Binding ability of quercetin, rutin and hesperidin was found to be higher for respective targets than the standard drug and presented in Figure 10, 11 and 12.

Table 2: Molecular binding energy of selected bioactive compounds against various targets

Targets (PDB ID)	Binding free Energy (Kcal/mol)				
	Diclofenac	Ferulic acid	Quercetin	Rutin	Hesperidin
IL17A (2VX)	**-7.0**	-5.7	-	-	-
IL4 (1ITL)	-6.2	-5.2	-	-	-
IFNG (7X45)	-5.5	-	**-6.1**	-	
IL1RN (1G0Y)	-7.8	-	**-8.8**	-	-
IL17A (1ITL)	-6.1	-	-	**-7.1**	-
IL10 (1INR)	-6.2	-	-	**-7.7**	-
NFKB1 (1K3Z)	-6.5	-	-	-	**-9.7**
FOXP3 (7TDW)	-7.0	-	-	-	**-9.4**

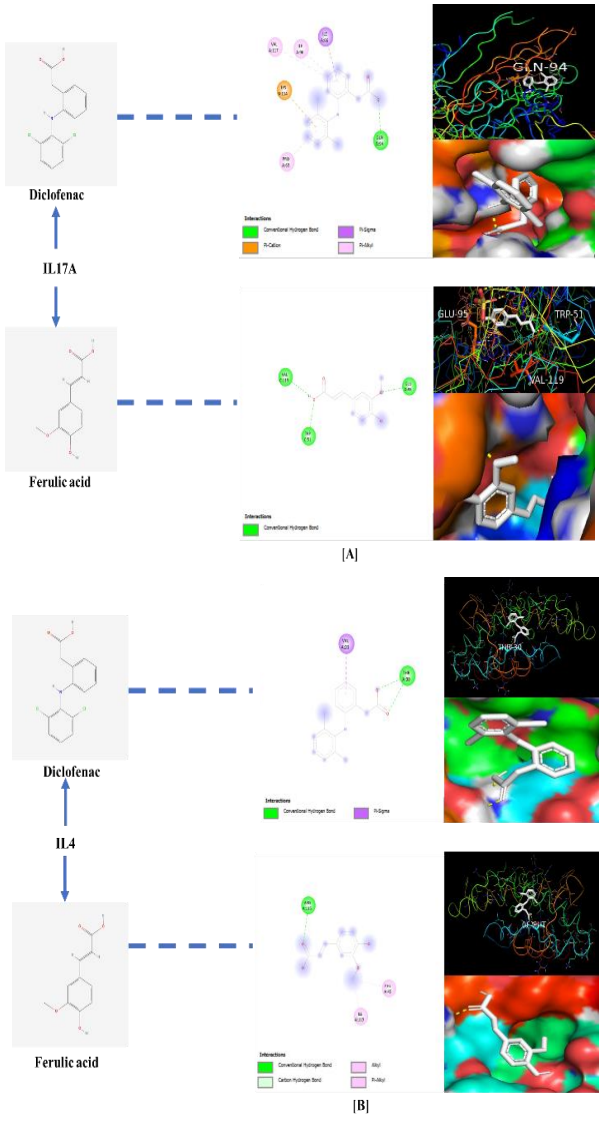


Figure 9. Binding free energy of diclofenac, ferulic acid against IL17A and IL4)

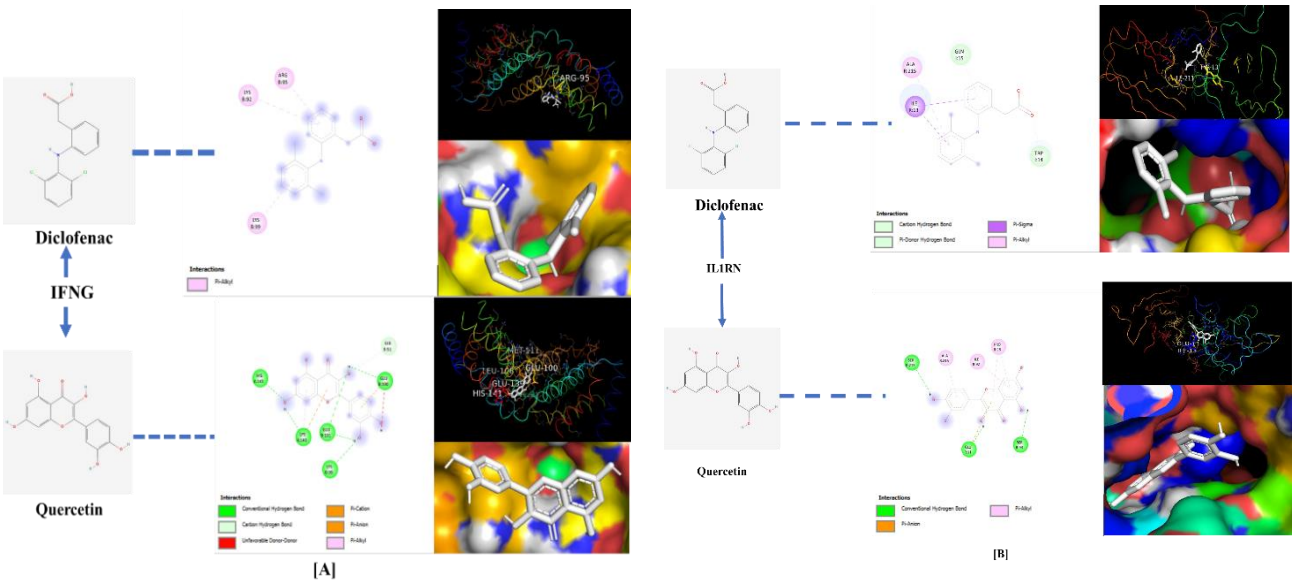


Figure 10. Molecular docking score of quercetin and diclofenac against IFNG and IL1RN

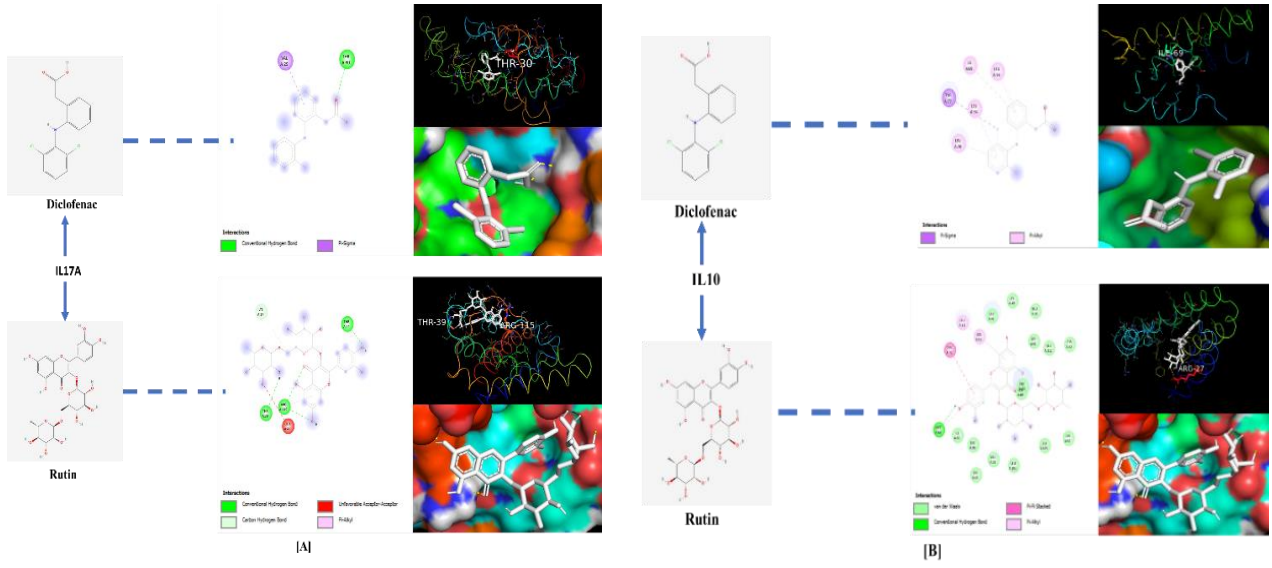


Figure 11. Binding free energies of protein (IL17A, IL10) & ligand (Diclofenac, Rutin)

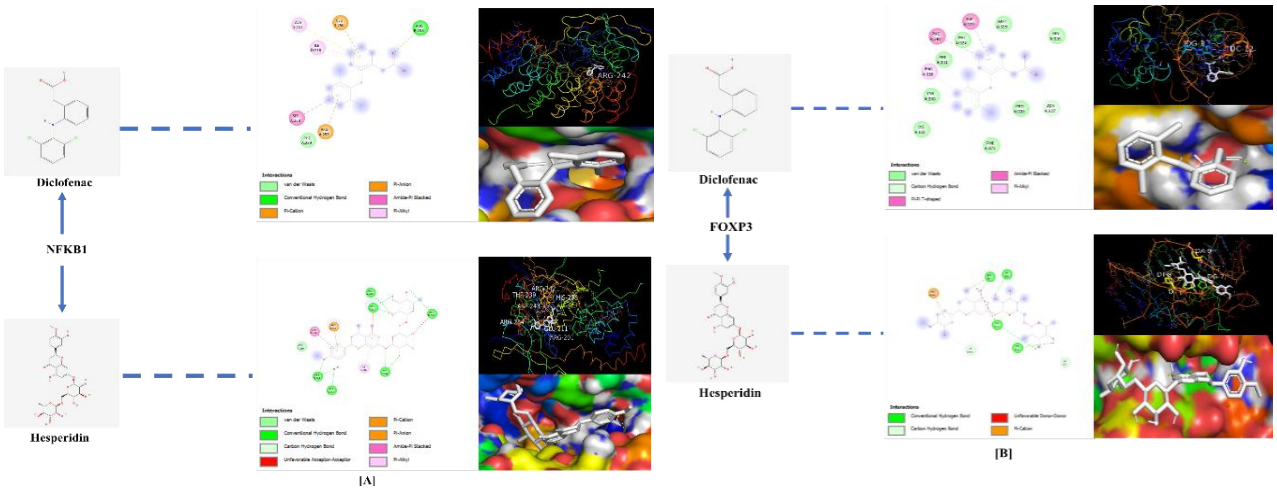


Figure 12. Docking score of protein (NFKB, FOXP3) with diclofenac and hesperidin

DISCUSSION

The inflammatory response is a key component of the body's defense mechanisms, involving a number of cellular and molecular activities. Chronic or excessive inflammation, on the other hand, can help to develop and progress a variety of diseases, including atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, asthma, cardiovascular problems, neurological ailments, autoimmune diseases, and cancer [37, 38]. The use of complementary therapies for treating inflammation is growing by the day, and it frequently goes unreported by physicians [39]. Flavonoids and phenolics are a possible option for developing inflammation-targeted treatments. Their ability to control inflammatory pathways, block pro-inflammatory enzymes, and exert antioxidant effects make them promising candidates for future research and usage in the treatment of inflammatory disorders [40- 42]. As a result, we apply network pharmacology, molecular docking analysis, and ADMET characteristics in this study to evaluate the influence and mechanism of action of flavonoids such as hesperidin, quercetin, rutin, and ferulic acid on inflammation.

Network pharmacology is an interdisciplinary field that combines concepts from pharmacology, network biology, and systems biology to understand the complex interactions between drugs and biological systems [43]. It involves the study of drug-target interactions, signaling pathways, and the overall network of molecular interactions within a biological system. Cytoscape tool was used to analyze the network clusters or modules [44]. By grouping nodes with same characteristics or connectivity patterns, clustering techniques in Cytoscape can expose functional modules inside a network. The anti-inflammatory effect of flavonoids is associated with various pathways, as demonstrated by our investigation of the potential critical nodes IL4, IL 17A, IFNG, IL1RN, IL10, NFKB1, and FOXP3, Figure 8 A, B, C, and D illustrates these findings. Important targets for inflammation include IL4, IL 17A, IFNG, IL1RN, IL10, NFKB1, and FOXP3.

Numerous genes involved in immunological responses are either activated or repressed as a result of this interaction [45]. Inflammatory mediators are produced as a result of the IL17A receptor signaling pathway's primary activation of downstream signaling pathways. The two subunits of the IL-17 receptor, IL-17RA and IL-17RC, are interacting with IL-17A [46]. The activation of the receptor is started when IL-17A attaches to the extracellular domain of IL-17RA. Act1 (sometimes referred to as CIKS), an adaptor protein required for

downstream signaling, is recruited by IL-17RA. Act1 recruits and triggers TNF receptor-associated factor 6 (TRAF6), a crucial signaling protein. Activating TRAF6 activates mitogen-activated protein kinases (MAPKs), which include p38, ERK1/2, and JNK. TRAF6 is an E3 ubiquitin ligase that facilitates the attachment of ubiquitin molecules to target proteins. Numerous transcription factors, including AP-1 (activator protein 1) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), are phosphorylated and activated by these kinases [47]. Genes related to inflammation and immunological responses, including NLRP3, Pro-IL-1β, and Pro-IL-18, are expressed when NF-κB translocates into the nucleus [48].

The immune system's reaction to inflammation and cell death are intricately linked to interferon gamma (IFN-γ) [49]. The Th1 subset is formed when CD4+ T cells differentiate into IFN-γ. Th1 cells are crucial in eliminating intracellular infections because they generate IFN-γ and stimulate macrophages. There are several ways that IL-1RN can signal, including intracellular signaling pathways that are started when IL-1RN binds to the IL-1 receptor on the cell surface. The activation of anti-inflammatory signaling pathways may ensue from this [50]. Interleukin-10, or IL-10, is a significant cytokine that has a role in both immunological control and anti-inflammatory reactions. The IL-10 receptor is composed of two subunits: IL-10R1 and IL-10R2. IL-10R1 is specific to IL-10, while IL-10R2 is shared with other cytokine receptors. IL-10 binds to the IL-10R1 subunit, leading to the recruitment and activation of IL-10R2 [51].

After IL-10 binding, the IL-10 receptor-associated JAK1 and Tyk2 (Janus kinases) are activated. JAKs are enzymes that phosphorylate tyrosine residues on the receptor subunits, initiating downstream signaling events. Activated JAKs phosphorylate and activate STAT proteins, particularly STAT3 [52]. Once activated, STAT3 proteins form dimers and translocate to the nucleus. In the nucleus, STAT3 dimers bind to specific DNA sequences known as STAT response elements (SREs) in the promoter regions of target genes. This binding leads to the transcription of various anti-inflammatory and immunosuppressive genes, including IL-10 itself, SOCS3 (Suppressor of Cytokine Signaling 3), and other downstream effectors. In the nucleus, STAT3 dimers bind to specific DNA sequences known as STAT response elements (SREs) in the promoter regions of target genes. This binding leads to the transcription of various anti-inflammatory and immunosuppressive genes, including IL-10 itself, SOCS3 (Suppressor of Cytokine Signaling 3), and other downstream effectors (Figure 13) [53].

NF-κB regulates the expression of various pro-inflammatory genes, including cytokines (e.g., IL-6, IL-8, TNF-α), chemokines, adhesion molecules, and enzymes involved in the production of reactive oxygen species [54]. These gene products contribute to the recruitment and activation of immune cells, increased vascular permeability, and tissue remodeling, all of which are key features of the inflammatory response. NF-κB pathway is regulated by negative feedback mechanisms to avoid excessive or persistent inflammation. Among these mechanisms are the cytoplasmic sequestration of NF-κB by IκBα resynthesis and the generation of anti-inflammatory molecules (such IL-10) that prevent NF-κB activation [55]. The regulation of immunological responses and the reduction of inflammation are intimately linked to FOX3 signalling. Tregs are the main cells that express FOX3, which is necessary for the formation and suppressive action of these cells. It acts as a master regulator by controlling the expression of various genes involved in Treg differentiation and function [56].

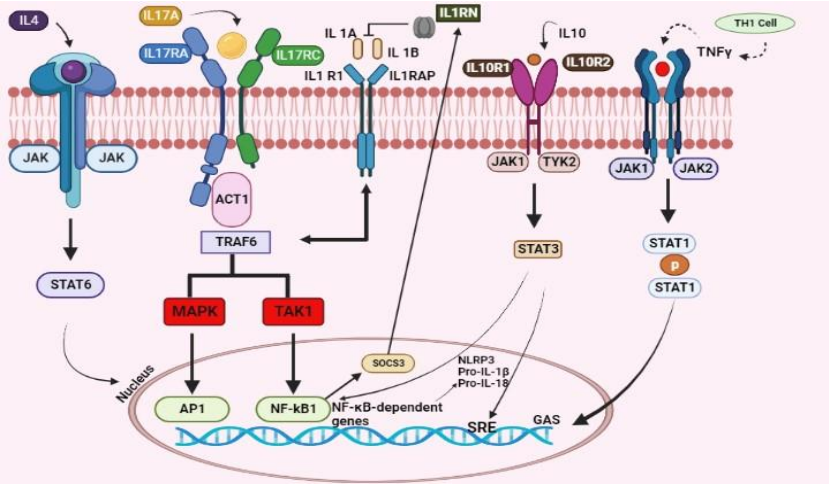


Figure 13. Inflammatory and anti-inflammatory signaling pathways of key targets identified from bioactive compounds

CONCLUSION

The current study reveals that chosen flavonoids (viz., ferulic acid, quercetin, rutin, and hesperidin) have a promising binding with diverse inflammatory targets, revealing their beneficial anti-inflammatory potential. According to the molecular docking results, the ligand exhibits a high affinity for the target protein. These findings provide important insights into the likely method of binding and can be used to validate and optimize the ligand as a potential medicinal agent. This virtual screening of selected flavonoids benefits many scholars in their future studies on the anti-inflammatory potential of natural compounds. It additionally serves as the foundation for future in-vivo and clinical trials on these natural flavonoids.

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Conflicts of interest

The authors declare that there is no conflict of interest.

Abbreviations

IL4: Interleukin-4; **IL6**: Interleukin-6; **IL18** Interleukin-18; **IL10**: Interleukin-10; **IL17A**: Interleukin-17A; **NFKB1**: Nuclear factor kappa B subunit 1; **FOXP3**: Forkhead box protein 3 gene; **IRA**: Inflammatory receptor agonist; **TNF**: Transforming growth factor; **TGF**: transforming growth factor; ADME-T: Absorption, distribution, metabolism, excretion and toxicity; GO: Gene ontology; **KEGG**: Kyoto encyclopedia of genes and genomes; **PPI**: Protein–protein interaction; **BP**: Biological process; **MF**: Molecular function; **CC**: Cellular function; **HIA**: passive gastrointestinal absorption; **BBB**: blood-brain barrier; **JAK/ STAT**: Janus kinase/signal transducers and activators of transcription; **TYK2**: Tyrosine-Kinase 2; **ACT**: Adaptor protein; **AP1**: activator protein 1; **MAPK**: Mitogen-activated protein kinase; **TAK1**: Transforming growth factor-β (TGF-β)-activated kinase 1; **SOCS3**: Suppressor of cytokine signaling 3; **NLRP3**: nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3; **TRAF6**: Tumor necrosis factor receptor associated factor 6.

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استكشاف الأهداف المضادة للالتهابات في الفلافونويدات من خلال الالتحام الجزيئي المتكامل وعلم الأدوية الشبكي

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ملخص

الالتهاب هو استجابة فسيولوجية معقدة مرتبطة بالعديد من الأمراض. أظهرت الفلافونويدات، وهي فئة من المركبات الطبيعية المنتشرة على نطاق واسع في النباتات، خصائص مضادة للالتهابات واعدة. ومع ذلك، تظل آليات عملها الشاملة وأهدافها الجزيئية المحتملة غير قابلة للتعريف. في الدراسة الحالية، استخدمنا نهج علم الأدوية الشبكي جنبًا إلى جنب مع الالتحام الجزيئي للتحقيق في التأثيرات المضادة للالتهابات لبعض الفلافونويدات. في البداية، قمنا بجمع وتنظيم قاعدة بيانات شاملة مثل معلمات وأهداف ADMET من ADME السويسرية وADMET 2.O وتوقع الهدف السويسري. ثم قمنا ببناء شبكة تفاعل بروتين–بروتين لتحديد البروتينات الرئيسية المشاركة في الالتهاب باستخدام قاعدة بيانات السلسلة. بعد ذلك، قمنا بدمج مجموعة بيانات الفلافونويد مع شبكة البروتين لتحديد تفاعلات الفلافونويد–البروتين المحتملة باستخدام Cytosacpe vina. تم إجراء تحليل إثراء GO وKEGG بمساعدة قاعدة بيانات David. تم إنجاز الالتحام الجزيئي من خلال Autodock Vina، وتقييم تقارب ارتباط الفلافونويدات المحددة تجاه البروتينات المستهدفة المحددة. قدم تحليل الالتحام رؤى حول التفاعلات المحددة بين الفلافونويدات والبروتينات المستهدفة، موضحًا الآليات المحتملة الكامنة وراء تأثيراتها المضادة للالتهابات. تعمل المكونات النشطة بيولوجيًا حمض الفيروليك، والكيرسيتين، والروتين، والهسبيريدين على تعديل العديد من العمليات الجزيئية والخلوية ثم تمارس تأثيرات مضادة للالتهابات. من التحليل، شاركت الأهداف الرئيسية في مرض التهاب الأمعاء، ومسار إشارات IL 17، ومسار إشارات TNF، ومسار إشارات بوساطة السيتوكين، والتهاب المفاصل الروماتويدي، والليبوبوليساكاريد وما إلى ذلك. كشفت دراسات الالتحام الجزيئي الإضافية أيضًا أن تقارب الارتباط للفلافونويدات المحددة كان أعلى من تقارب الديكلوفيناك.

الكلمات الدالة: مضاد للالتهابات؛ حمض الفيروليك؛ الهسبيريدين؛ الالتحام الجزيئي؛ علم الأدوية الشبكي؛ كيرسيتين؛ روتين.

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A Recent Review of PLGA-PEG Hybrid Nanoparticles for Anticancer and Anti-Inflammatory Applications

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ABSTRACT

Numerous synthetic polymers have been investigated to be used in nanomedicine over the past few decades, particularly in drug delivery systems. Necessitating properties including non-toxic, biodegradable, and biocompatible. Among these, polylactic-co-glycolic acid (PLGA) which stands out due to its complete biodegradability and ability to self-assemble into nanometric micelles. However, their large diameter (150–200 nm), poor stability in aqueous media, and their removal from the bloodstream by the liver and spleen hindering the *in vivo* treatments. Polyethylene glycol (PEG) is the most widely used polymer in drug delivery systems, and the first PEGylated product has been on the market for over 20 years. PEG has a stealth behavior; therefore, it will not be recognized by the immune system. Further, PEG is hydrophilic polymer that could stabilize nanoparticles through steric rather than ionic effects. In this review article, the important of utilizing PLGA-PEG nanoparticles as polymeric drug carriers has been revised and the advantages of employing PLGA-PEG copolymer to form stable and well-defined, nanoparticles for drug delivery applications have been summarized. Moreover, the review aimed to shed light on the various methods employed in their preparation. Additionally, recent advancements in PLGA-PEG copolymer preparations for anti-cancer and anti-inflammatory therapies, are discussed in detail. The other applications of PGA-PEG have been extensively reviewed in other publications. Therefore, it was not addressed in this review.

Keywords: Nanotechnology; PLGA NP; Medical application; Cancer; Anti-inflammatory.

1. INTRODUCTION

In recent years, biomaterials research has been intensively growing, particularly in utilizing the nanoparticles (NPs) as a drug delivery system, which possess great potential for technical breakthroughs in the field of drug delivery. The use of nanoparticles has been evaluated in-depth and has been driven by the ability to deliver a wide range of materials, including hydrophilic and hydrophobic drugs, vaccines, and biological macromolecules. Nanoparticles also allow targeted delivery of drugs to specific organs or site within the body [1-3]. Polymeric NPs have shown promise as an innovative approach for drug delivery application. It is worth noting that approximately >80% of new drug candidates are categorized as poorly water-soluble, as reported by Wulff-Pérez et al. In 2014.[4].

Consequently, these drugs often face challenges such have a low absorption, short circulation time and un-wanted side effects. However, using polymeric NPs can effectively address these issues. In addition, NPs exhibit various properties depending on their size and hydrophilicity. Their small size enable them to penetrate deep into tissue, reaching the target site (Table 1) [5]. Furthermore, polymeric NPs have the ability to selectively bind to site of action with minimal side effects as well

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avoiding harming to other cells. This can be achieved by functionalizing their surface with specific proteins, peptides, monoclonal antibodies thus enhance their efficiency [6-8].

It is important to note that polymers utilized in nanomedicine must adhere with a standard set by the Food and Drug Administration's (FDA) including various criteria including biocompatibility, safety, good drug loading efficacy, good mechanical properties, and well-characterized structure. Whilst numerous synthetic polymers have been employed to create NPs, it's noteworthy that not all are appropriate for application in the nanomedicine [9]. Among these synthetic biodegradable polymers are polyamides, polyesters, poly (amino acids), polyurethanes which have been increasing utilized in the past two decades due to their controllable properties in terms of molecular weight and shape [3, 10].

Table 1: Comparison of Smart PEG-PLGA Hybrid NPs with Different Nanoparticles [11, 12]

Criteria	PEG-PLGA Hybrid NPs	Liposomes	Polymeric NPs	Metallic NPs
Composition	PEG-PLGA	Lipid bilayers	Various polymers (PLGA, PCL, PVA…)	Gold, silver…
Size Range	50 - 200 nm	50 - 200 nm	10 - 200 nm	1 - 100 nm
Surface Functionalization	PEGylation, Ligand Conjugation	Phospholipid functionalization	Polymer coating	Surface modification
Drug Loading Capacity	High	Variable	High	Moderate to High
Controlled Release	Yes	Yes	Yes	Yes
Biocompatibility	High	Generally high	Variable	Variable
Stability	Good	Moderate to High	Variable	Moderate to High
Targeting Capabilities	Active and Passive Targeting	Active and Passive Targeting	Active and Passive Targeting	Active Targeting
In Vivo Performance	Efficacy and Safety	Proven efficacy	Varied depending on polymer	Variable
Imaging Modalities	Fluorescent, MRI, CT, etc.	Limited imaging capabilities	Limited imaging capabilities	CT, MRI, Photoacoustic
Toxicity	Low	Generally low	Variable	Variable
Clinical Translation	Promising	Established in some cases	Under investigation	Limited
Challenges and Limitations	Limited Payload, Manufacturing	Stability, Batch Consistency	Burst release, Biodegradability	Aggregation, Biodistribution
Future Perspectives	Advancements and Innovations	Integration with imaging	Tailoring for specific drugs	Combined therapies

Among these biodegradable polymers is poly (lactic-co-glycolic acid) (PLGA), which is made up of two cyclic dimers of glycolic and lactic acid copolymerized randomly by ring-opening (Figure 1) [3, 13]. The mole ratio of glycolic to lactic acid in the polymer chain has a crucial role in determining the properties of PLGA, such as the degree of crystallinity, mechanical strength, swelling behavior, and hydrolyzing capacity. This, in turn, affects the rate of biodegradation [14]. However, their large diameter (150–200 nm), poor stability in aqueous media, and their removal from the 2bloodstream by the liver and spleen hindering in vivo treatments.

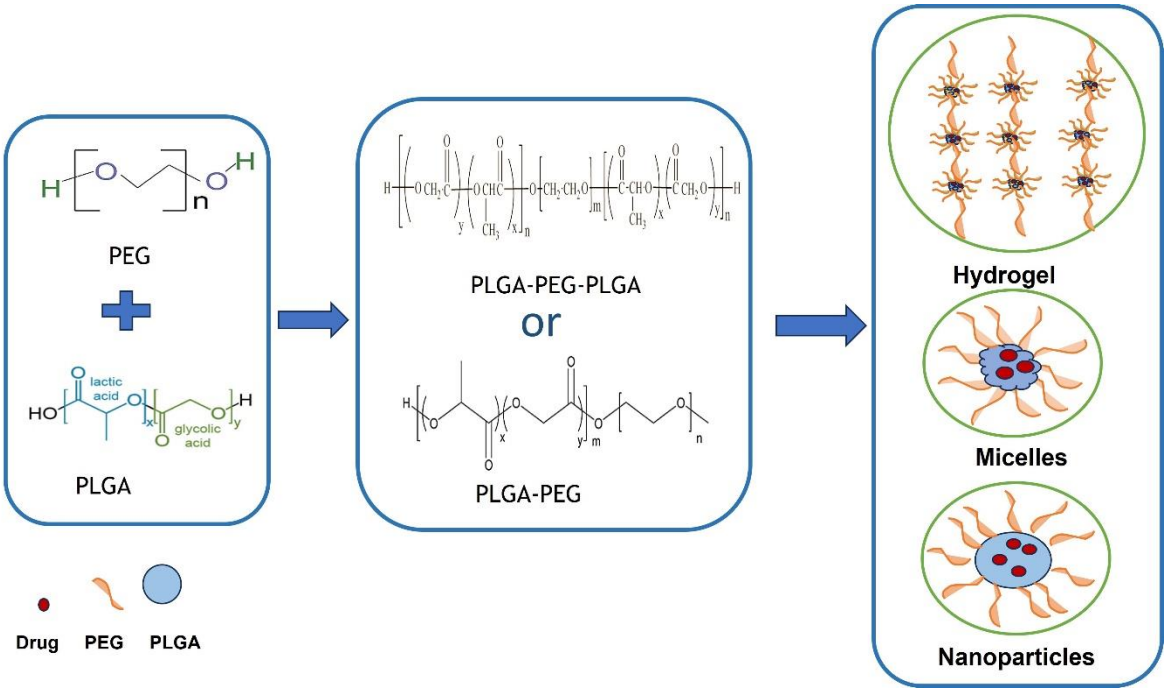

Figure 1: Scheme represents PLGA-PEG nanoparticles formation technique using probe sonication

The biodegrading process of PLGA starts in an aqueous medium, where PLGA completely biodegrades to its original monomers, lactic and glycolic acids, which then the body can either excrete them unchanged in the kidney or metabolize them and eliminate carbon dioxide and water ensuring a safe clearance of PLGA out of the body [14].

PLGA-derived systems, and the first PEGylated product has been on the market for over 20 years. can be encapsulate various drugs, proteins, and peptides that are released gradually, in a time -dependent manner in the body [15]. However, it should be noted that these systems come with significant limitations. Firstly, a stabilizer such as poly (vinyl alcohol) must be used to ensure stability of nanoparticles achieving small diameter with uniform distribution. Additionally, PLGA NPs are readily recognized by reticulo-endothelial system (RES) which is composed of the liver and spleen. This identification leads to eliminate PLGA NPs from blood circulation and significantly shortens their circulation residence time thereby NPs delivered to specific organs or tumor tissues [16].

To date, the potential applications of polyethylene glycol (PEG) with molecular weight of 300 to100,000 Da is highly encouraging, since PEG is not a biodegradable polymer, PEG has a stealth behavior; does not accumulate

in tissue and is eliminated from the body without undergoing biodegradation processes [17]. This particularly comes with those with low molecular weight chains. Furthermore, PEG is widely used in medicine for a variety of purposes such as a laxative agent, excipient in drug formulation as well a coating agent particularly in capsule formulations [18, 19]. Moreover, PEG's hydrophilicity enhances solubility, prevents aggregation, and stabilizes nanoparticles in aqueous media could stabilize nanoparticles through steric rather than ionic effects thereby its worthy to use. Currently many researchers focused on finding new candidate drugs encapsulated within PLGA PEG based NPs due to their ability to encapsulate a variety of hydrophilic and hydrophobic agents [20-22] [23].

Interestingly, several studies investigated the possibility of conjugating the nanoparticles with linear PEG through a process known as PEGylation [24], aiming to improve their pharmacokinetic profile and lessen their toxicity. PEG is considered a promising candidate for adsorption or grafting on NP surfaces to escape clearance by the body's mononuclear phagocytic system (MPS), since they are neutral, hydrophilic along with their strong spatial repulsion characteristics [25, 26].

In this review article, the important of utilizing PLGA-PEG nanoparticles as polymeric drug carriers has been revised and the advantages of employing PLGA-PEG copolymer to form stable and well-defined, nanoparticles for drug delivery applications have been summarized. Moreover, the review aimed to shed light on the various methods employed in their preparation. Additionally, recent advancements in PLGA-PEG copolymer preparations for anti-cancer and anti-inflammatory therapies, are discussed in detail along with its role in the disease.

2. Synthesis of PLGA-PEG copolymer

Several synthetic approaches were developed to prepare different kinds of PLGA-PEG copolymers with various block structures and compositions. The biodegradation rate and hydrophilicity of PLGA-PEG copolymers can be modulated by adjusting the ratio of its hydrophilic and hydrophobic constituents. The properties of PLGA-PEG block copolymers are typically very different when compared to their constituent polymers, so they are developed into a new class of biomaterials with distinctive qualities of their own, including water solubility, crystallinity, microphase separation, and biodegradability[27].

Gref et al. (1994) initially described the synthesis of PLGA-PEG, featuring a covalent linkage between a PLGA chain carrying a free carboxylic acid at one end (PLGA-COOH) and a PEG chain functionalized at both ends with an amino. The resulting product undergoes repeated washing with organic solvent. This process leads to the formation of an amido group in the middle of the copolymer, which remains stable when stored in a freezer for several months [28, 29].

3. Preparation Methods for PLGA-PEG Nanoparticles

Various methods have been identified for PLGA-PEG NPs fabrication including double emulsion, nanoprecipitation, single-emulsion solvent-evaporation, solvent displacement technique, and the emulsification-solvent diffusion technique [30].

PLGA-PEG NPs can be prepared by double emulsion method. In this method PLGA–PEG was dissolved in ethyl acetate forming the organic phase. Simultaneously, the drug was dissolved in deionized water preparing the aqueous phase. The primary emulsion (w_1/o) was formed by using sonication energy (Figure 2). Subsequently, ultrasonic energy was used to produce a secondary emulsion ($w_1/o/w_2$), where the primary emulsion (w_1/o) was distributed in deionized water of polyvinyl alcohol (PVA). Following the evaporation of the solvent, the formed NPs were subjected for washing by centrifugation [31, 32].

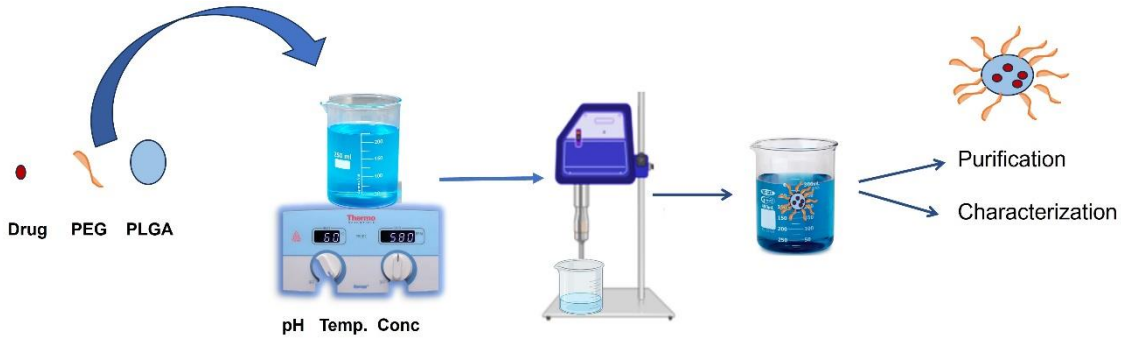

Figure 2 : Scheme represents PLGA-PEG nanoparticles formation technique using probe sonication

Nanoprecipitation is a versatile method that enabling the encapsulation of various therapeutic agents within the nanoparticles, making it suitable for drug delivery functions [30]. In this technique the drug was dissolved in organic layer that are miscible with water along with PLGA–PEG was dissolved and mixed with the drug by stirring. Subsequently, NPs were centrifuge and resuspended in water [33].

Likewise, PLGA–PEG NPs can be prepared by single-emulsion solvent-evaporation method. In this method the NPs were prepared by dissolving the drug, PLGA with PEG at room temperature. This organic phase was rapidly poured into PVA aqueous solution and then emulsified through sonication to form an oil-in-water (O/W) emulsion. Consequently, the organic solvent rapidly evaporated under the vacuum. The particles were then recovered by centrifugation and washed with water to eliminate the surfactant residue (PVA). The NPs were dispersed in the cryoprotectant sucrose, and the resulting nanosuspension was subjected to freeze-drier [34, 35].

Solvent displacement is another technique that can be used in PLGA-PEG NPs preparation. Initially, PLGA-PEG polymer was dissolved in acetone and the solution was gradually transferred dropwise into a stirred solution of sodium cholate in phosphate buffered saline. Subsequently, the acetone allowed to evaporate, and the resulting suspension of NPs was condensed in a rotary evaporator. Gel permeation chromatography was employed to purify the formed NPs [36].

At the men time, PLGA-PEG NPs can be prepared through-solvent diffusion technique. In this technique the polymer was dissolved in a suitable solvent while the drug was dissolved in water containing an emulsifying agent. This solution was slowly dropped at constant speed into liquid paraffin presented as an external phase. This process was conducted under homogenization utilizing high-speed homogenizer at various agitation speeds. The formed NPs were isolated by ultracentrifugation and the separated NPs were washed with n-hexane to remove residual liquid paraffin. Ultimately, a fine powder of NPs was obtained by lyophilization of the formed suspension [37].

4. Benefit and toxicity consideration of PLGA-PEG NPs:

PLGA-PEG polymer is considered a potent platform esteemed by scientists due to its biocompatibility, making it a valuable material for medical application. Although various materials are available for utilizing NPs, the majority of these still require FDA approval; in contrast, PLGA and PEG have already received this recognition, allowing their use in preclinical and *in vivo* testing. Furthermore, the PLGA-PEG copolymer can readily form micelles with well-controllable sizes and high encapsulation efficiencies without the aid of surfactant agents, which are frequently toxic and poorly tolerated by cells and organisms [38]. PLGA and PEGylated NPs were demonstrated the ability to improve the pharmacokinetic

profiles and biodistribution of several drugs. For instance, it was reported that the association of docetaxel with PLGA and PEGylated PLGA NPs modified the pharmacokinetics and biodistribution of docetaxel. Loading of docetaxel in NPs contributed to an increased blood residence time of docetaxel fulfilling NP's role as a long-circulating sustained-release drug delivery system. Also, surface modification of NPs, contributed to more pronounced blood concentrations of docetaxel, confirming the role of PEG in facilitating NPs escape from clearance out of the body [39].

Despite numerous benefits of using PLGA-PEG, some drawbacks have limited their widespread use. Primarily, the synthesis process of PLGA-PEG is considered costly and time-consuming. Both PLGA and PEG are synthetic polymers that require several steps for preparation, including industrial processes, purification procedures, and the use of costly building blocks. In contrast, natural polymers like chitosan may offer simpler and potentially more cost-effective alternatives. [40], are derived from natural waste material and do not require lengthy artificial processes for preparation. In addition, the autocatalysis of PLGA at low pH where the carboxylic end groups of degraded products can accelerate the degradation and decrease the local pH. Hence, this acidic environment limits their protein delivery and typically requires the incorporation of antacids, such as Mg $(OH)_2$, into the polymers for protein stabilization [41]. Moreover, heavy metals like stannous, which are used during the polymerization step of lactic and glycolic acid, must be eliminated from the polymer chains to mitigate any toxicity or organ damage [42].

5.1 PLGA-PEG NPs for cancer treatment:

Recently, there have been reports on the use of (PLGA-PEG NPs) for cancer targeted therapy. Interestingly, it was found that these delivery vehicles, designed to transport drugs precisely to specific targets within the body[43]. The PLGA part provides a substantial structure, while the PEG component ensures stealth-like properties, preventing the immune system from recognizing and attacking them Figure 3.

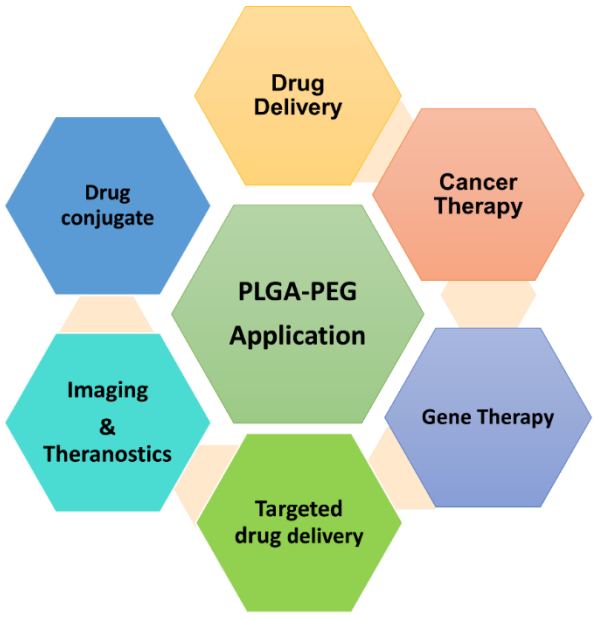

Figure 3: Representative scheme of PLGA-PEG hybrid copolymers applications.

This dynamic couple makes PLGA-PEG NPs versatile tools in the field of medicine, enabling more effective and targeted drug delivery with minimal side effects (Table 2). In essence, they act as microscopic superheroes, navigating our bloodstream to deliver the right medicine to the right place at the right time [44] [45].

Table 2: Examples of different drugs encapsulated into PLGA: PEG nanoparticles.

Drug	Composition and Ratio of PLGA: PEG	Indication	Study type	Refences
Docetaxel	PLGA (lactide/glycolide ratio of 50:50, carboxylic acid end group, molecular weight, 17,000 Da) PEG polymer with terminal amine and carboxylic acid functional group (NH2-PEG-COOH)	Prostate Cancers	In vitro Cytotoxicity and uptake	[46]
Docetaxel	PLGA– PEG diblock copolymer (50:50 PLGA attached to mPEG 5000, 15%. wt)	Cancer	In vitro In vivo (Kinetics)	[47]
Curcumin	PLGA-PEG (50:50 lactide:glycolide, PVA (1%, w/w	Breast cancer	In vitro MCF7 breast cancer cell line	[48]
Dactolisib	blend of poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide-co-glycolide)-poly(ethylene glycol)-2000 (PLGA-PEG)	Targeted delivery of the mTOR/PI3kinase inhibitor to inflamed endothelium	In vitro, tumor necrosis factor-α (TNF-α) activated endothelial cells	[49]
Cisplatin	PLGA-PEG (50:50 lactide:glycolide, PVA (1%, w/w	cancer cells	In vitro A549 lung	[50]
Doxorubicin and Tetrahydrocurcumin	PLGA-PEG (50:50 lactide:glycolide)	Glioma	In vitro, MTT Ex vivo DOX fluorescence imaging. The in vivo tumor orthotopic C6 mouse models	[51]
Metformin and Silibinin	poly (D, l -lactide-co-glycolide)-polyethylene glycol	Breast cancer	In vitro T47D breast cancer cell line	[52]
Cyclodextrin-Peganum harmala Alkaloid Complex and Ascorbic Acid	Poly (D,L-lactide-co-glycolide) (PLGA) and polyethylene glycol 6000 (PEG), Polyvinyl alcohol (PVA; 98% MW ≈ 13,000)	Cancer	In vitro Cytotoxicity	[53]
Honokiol	PLGA-PEG (50:50 lactide:glycolide)	Anti-tumour activities	Stability study	[54]
Ciprofloxacin	(Average MW of PEG 2000 g/mol), PLGA (average MW of 11,500 g/mol) (lactide: glycolide=50:50	Bactericidal activity was observed against Enterococcus faecalis	In vitro	[55]
The A10 PSMA aptamer for in vivo targeted drug delivery	Carboxy-terminated poly(D,L-lactide–**co**–glycolide)–**block–** poly(ethylene glycol) (PLGA–**b**–PEG–COOH)	Prostate cancer	The in vivo (xenograft mouse model of prostate cancer)	[56]

Table 1 provides a summary of *in vitro* and preclinical studies that utilized PEG-PLGA in cancer application. Starting with breast cancer, metformin an anti-hyperglycemic agent was encapsulated into folate-functionalized PLGA- NPs, showing a higher cytotoxic effects with remarkable down-regulation of hTERT, Bcl-2 and up-regulation of Caspase7, Caspase3, Bax, and p53 gene expression in breast cancer MDA-MB-231 cell line, therefore these NPs are suggested as an appropriate approach to elevate the anticancer properties of metformin for improving the treatment effectiveness of breast cancer cells [57]. Similarly, treatment with metformin and Silibinin co-loaded in PLGA-PEG NPs resulted in synergistic anticancer activity against T47D breast cancer cells. Also, this dual drug-loaded NPs revealed a significant changes in the expression levels of Bax, Bcl-2, caspase 3 and hTERT compared to the single drug-loaded NPs. As a result, treatment with PLGA-PEG NPs based on combination therapy might have a significant potential to improve the efficiency of breast cancer treatment [58].

In another study, curcumin was loaded into epidermal growth factor receptor (EGFR)-targeting GE11 peptides conjugated with PLGA nanoparticles. This loading was revealed to reduce phosphoinositide 3-kinase cascade and decrease cancer cell proliferation, attenuated drug clearance from the blood circulation, and repressed tumor burden compared with free curcumin or non-EGFR targeting nanoparticle.

In case of colon cancer, S. Khaledi et al (2020) was prepared PLGA-PEG-PLGA NPs loaded 5-FU and Chrysin, a natural compound using double emulsion method. The study reported that the 5-FU and Chrysin with PLGA-PEG-PLGA copolymer exhibited a remarkable uptake in HT29 cells and were found to have significant effect on the cell proliferation compared with NPs loaded with each drug alone in HT29 cell line. In addition, the synergistic anticancer effects of 5-FU and Chrysin in NPs were loaded with a clearance value of 0.35 [59].

For prostate cancer, Dhar et al. (2008) utilized a PLGA-PEG copolymer to create cisplatin-loaded nanoparticles, which were specifically targeted using a prostate-specific membrane antigen (PSMA) aptamer. The study demonstrated that these targeted nanoparticles effectively impacted human prostate cancer cells overexpressing PSMA (LNCaP and PSMA-PC3) while having no effect on normal cells. Additionally, the targeted nanoparticles increased cell death by ten times compared to non-targeted nanoparticles. [60]. In a related study, Farokhzad et al. (2006) developed PLGA-PEG nanoparticles designed to target PSMA and loaded with docetaxel. These nanoparticles specifically bound to prostate cancer cells and demonstrated an increased cytotoxic effect of docetaxel. Furthermore, in vivo experiments with xenograft nude mice bearing tumors showed that a single injection of these nanoparticles resulted in complete tumor volume reduction. Remarkably, 100% of the treated mice survived after 109 days, compared to only 14% of the untreated mice. [61].

Another study involved a lung cancer model using Calu-6 lung adenocarcinoma cells. In this study, siRNA was encapsulated into iron magnetic nanoparticles (MNPs) modified with PLGA and PEG. The results showed that the expression of the telomerase gene was significantly lower in the cells treated with siRNA-magnetic copolymers compared to those treated with free siRNA in the lung cancer cell line. [62]

5.2 PLGA-PEG NPs as a delivery system for anti-inflammatory:

Recently, PLGA-PEG nanoparticles have shown significant potential in enhancing the therapeutic efficacy of anti-inflammatory drugs, making them a promising delivery system for treating inflammatory conditions and diseases. For example, PLGA-PEG polymers can be used to synthesize nanoparticles for targeted delivery to specific cell types within the airways, particularly in obstructive lung diseases [63]. Additionally, Vij et al. (2016) encapsulated ibuprofen into PLGA-PEG nanoparticles, which were further modified with a maleimide-capped PEG to reduce the neutrophil-mediated inflammatory response in COPD. This approach leverages

the mucus inertness of PEG and the coupling properties of maleimide to achieve effective penetration and targeting of the required tissue. [64]. The findings indicated that these nanoparticles, following airway transport, could specifically bind to and release the drug into neutrophils, thereby controlling COPD inflammation. This formulation significantly reduced inflammation compared to the saline serum control 210 minutes after inflammation initiation. Additionally, it reduced ocular inflammation, suggesting it could be a promising candidate for topical application. [65].

The PLGA-PEG polymer has also been utilized for the controlled delivery of anti-inflammatory drugs. Gusperimus, an immunosuppressive drug used for autoimmune diseases and to prevent organ transplant rejection, was encapsulated in PLGA-PEG nanoparticles to enhance drug stability and bioactivity. [43, 66]. In a similar study, Li et al. prepared dexamethasone-loaded PLGA-PEG nanoparticles. These nanoparticles improved the pharmacokinetic properties of dexamethasone and reduced its side effects. When combined with ultrasound (US), dexamethasone-PLGA-PEG nanoparticles may offer a promising drug delivery system to enhance the therapeutic effects of dexamethasone. [67]. Another finding supported that PLGA-PEG nanoparticles exhibit increased interaction with Caco-2 cells and show superiority in inflamed cells. This interaction was further enhanced by increasing the particle size and polydispersity index. [68].

Table 3 summarizes the *in vitro* studies of the role of PLG-PEG in inflammatory diseases.

Drug	Design study	Main finding	Reference
BRP-201	PEG–Lipid–PLGA were prepared using DSPE–PEG–Lipids	It tested on the pro-inflammatory M_1-MDMs and mmn g shown to inhibit 5-LOX product formation and thus inflammation by delivering BRP-201 to the intracellular in 15 min or less.	[69]
Gusperimus	PLGA-PEG NPs	PLGA-PEG/Gusperimus nanoparticles were taken up by macrophages and exerted anti-inflammatory effects as it is indicated by nitric oxide reduction and cytokine suppression in LPS-induced inflammatory macrophages model.	[70]
PS-341 (bortezomib)	PLGA-PEGPS-341	The drug release in CF mice lungs was established by quantifying the changes in proteasomal activity with~2 fold decrease and capability to rescue the Pseudomonas aeruginosa that induced inflammation, which proved the rescue of CF lung disease in murine model.	[71]
Licochalcone-A	PLGA have been utilized PEG and cell penetrating peptides (Tet-1 and B6).	Reduce inflammation compared to the saline serum control in a significant manner after 210 min of inflammation initiation, also it was reducing the ocular inflammation.	[72]

Drug	Design study	Main finding	Reference
Magnolol polyphenol extracted from magnolia plant	magnolol-encapsulated (PLGA-PEG) nanoparticles	This formulation significantly reduced airway hyperresponsiveness, lung tissue eosinophil infiltration, and levels of IL-4, IL-13, TGF-β1, IL-17A, IgE and IgG1 in OVA-exposed mice compared to control-treated mouse also it prevented mucus overproduction and collagen deposition *in vivo* .	[73]
Teicoplanin	mPEG-PLGA hydrogel copolymer as a sol-gel drug delivery system for treating bone infection.	Implantation of the mPEG-PLGA hydrogel containing teicoplanin was effectively treating osteomyelitis in rabbits using histological staining and immunoblotting test.	[74]
recombinant Amb a (*Ambrosia artemisiifolia)*	Amb a 1 -loaded PLGA-PEG nanoparticles.	These nanoparticles enhanced the secretion of T-helper 1 (Th1) cytokine Interferon-gama (IFN-γ) and the production of immunoglobulin G, inhibited the secretion of T-helper 2 cytokine Interleukin 13, 4 and the level of IgE.	[75]
Dactolisib (mTOR/PI3 K inhibitor	Dactolisib nanoparticles composed of (PLGA-PEG) prepared by an oil/water emulsion solvent evaporation method	Dactolisib nanoparticles have a high potential reaching inflamed endothelial cells. E-selectin targeted nanoparticles loaded with Dactolisib had a pronounced effect on inflammation-activated endothelial cells as compared to the non-targeted NPs.	[49]
etoricoxib	PEG-PLGA-Nps using emulsion solvent evaporation approach	In-vivo study shows that PEG-PLGA etoricoxib decreases the swelling index and number of writhes in rat model. Also it remarkably increased the bioavailability of etoricoxib through oral dosage form.	[76]

6. The limitations and challenges of using PLGA-PEG nanoparticles

Despite the remarkable widespread use of PLGA-PEG in drug delivery applications several challenges remain. However, they face several challenges and limitations. One major issue is the potential for burst release, where a large amount of the encapsulated drug is released rapidly, reducing therapeutic efficacy and increasing side effects [77]. Additionally, the biodegradation rate of PLGA can be inconsistent, influenced by factors like particle size, copolymer ratio, and environmental conditions, complicating controlled drug release [78]. PEGylation can improve circulation time but may induce an immune response or accelerate clearance in some cases Manufacturing these nanoparticles at scale while maintaining consistency and reproducibility also poses significant technical hurdles [79, 80]. Understanding and addressing these challenges is crucial for the successful

clinical translation of PLGA-PEG nanoparticles [81]. Indeed, it's important to understand the biodegradation kinetics profile of PLGA-PEG *in vivo* to ensure their efficacy and confirm their safety. Also investigating the synergistic effect for combination therapies using NPs, that offer potential effects and minimizing toxicity should be explored.

To mimic the effects of nanoparticles (PLGA-PEG NPs) on cancer tumors or inflammation in patients, clinical studies involving regulatory agencies are crucial for addressing toxicity and efficacy. However, performing clinical trials is challenging and costly compared to *in vitro* studies. Scale-up and manufacturing steps of PLGA-PEG nanoparticles production is crucial but its cost-effective and required scalable production approaches to be widespread. Overall while PLGA-PEG nanoparticles hold a great value in drug delivery and biomedical applications, further interdisciplinary research and studies with different drugs and cell lines still required to explore and more understand their role in different cancer cell types.

7. Summary and Future Directions

In summary, this review underscores the promising role of PLGA-PEG nanoparticles (NPs) in drug delivery, especially in treating cancer and inflammation. These nanoparticles offer benefits like being safe for the body, releasing drugs in a controlled way, and improving how drugs move in the body. Studies show they can effectively deliver various drugs, enhancing treatment results in lab and animal tests.

However, challenges remain. One big concern is how quickly drugs are released from PLGA-PEG NPs, which can affect how well they work and their side effects. Also, how quickly PLGA breaks down and possible immune reactions to PEG need careful consideration for use in people. Making these nanoparticles on a large scale and keeping them consistent are also tough.

Looking ahead, future research should focus on solving these challenges and making PLGA-PEG NPs even better. Improving how drugs are packed into these nanoparticles, controlling how drugs are released, and making sure they target the right cells are key goals. It's also important to study how these nanoparticles work with different diseases and cell types.

To make PLGA-PEG NPs available for patients, more studies in people are needed to check safety and how well they work. This step is crucial for getting approval from health regulators and using them in real medical treatments. Teamwork between scientists, doctors, and companies will be crucial to overcome challenges and fully use PLGA-PEG nanoparticles to treat diseases more effectively.

In conclusion, while PLGA-PEG nanoparticles show great potential for improving drug treatments, ongoing research and new ideas are vital to make them work even better and solve current problems.

The important hallmarks of PLGA-PEG NPs are drug loading capacity, chemical targeting potential, and improved circulation time, which makes this material very attractive in the drug delivery industry. In summary, PLGA-PEG nanoparticles stand as established nanocarriers suitable for several nanomedicine purposes. These NPs have substantial attention, particularly for emerging anticancer and anti-inflammatory applications. This is largely due to their remarkable physicochemical properties. Various techniques have been involved for the preparation of PLGA-PEG NPs.

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مراجعة حديثة للجسيمات النانوية الهجينة (PLGA-PEG) للتطبيقات المضادة للسرطان والمضادة للالتهابات

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ملخص

تمت دراسة العديد من البوليمرات الاصطناعية لاستخدامها في الجسيمات النانوية على مدى العقود القليلة الماضية، وخاصة في أنظمة توصيل الأدوية. تتطلب خصائصها بما في ذلك غير سامة، والقابلة للتحلل، ومتوافقة حيويا. ومن هذه العناصر، حمض الجليكوليك المتعدد PLGA الذي يتميز بقابليته للتحلل البيولوجي الكامل وقدرته على التجميع الذاتي في الجسيمات النانوية. ومع ذلك، فإن قطرها الكبير (150–200 نانومتر)، وضعف ثباتها في الوسائط المائية، وإزالتها من مجرى الدم عن طريق الكبد والطحال، يعيق العلاجات داخل الجسم الحي. يعد البولي إيثيلين جلايكول PEG هو البوليمر الأكثر استخدامًا على نطاق واسع في أنظمة توصيل الأدوية، وأول منتج مصنوع من البولي إيثيلين موجود في السوق منذ أكثر من 20 عامًا. PEG لديه سلوك خفي. وبالتالي لن يتعرف عليه الجهاز المناعي. علاوة على ذلك، فإن PEG عبارة عن بوليمر محب للماء يمكنه تثبيت الجسيمات النانوية من خلال التأثيرات الاستاتيكية بدلاً من التأثيرات الأيونية. في هذه المقالة المراجعة، تمت مراجعة أهمية استخدام الجسيمات النانوية PLGA-PEG كحاملات للأدوية البوليمرية وتم تلخيص مزايا استخدام البوليمر المشترك PLGA-PEG لتشكيل جسيمات نانوية مستقرة ومحددة جيدًا لتطبيقات توصيل الأدوية. علاوة على ذلك، هدفت المراجعة إلى تسليط الضوء على الأساليب المختلفة المستخدمة في إعدادها. بالإضافة إلى ذلك، تتم مناقشة التطورات الحديثة في مستحضرات البوليمر المشترك PLGA-PEG للعلاجات المضادة للسرطان والمضادة للالتهابات بالتفصيل. تمت مراجعة التطبيقات الأخرى لـ PGA-PEG على نطاق واسع في منشورات أخرى. ولذلك، لم يتم تناولها في هذه المراجعة.

الكلمات الدالة: تقنية النانو، PLGA NP، التطبيقات الطبية، السرطان، مضادات الالتهاب.

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Exploration of Antidiabetic and Diuretic Activities of *Lumnitzera racemosa* and *Eclipta alba* with Molecular Docking Study

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ABSTRACT

The goal of the current study was to look at the leaves of *Lumnitzera racemosa* (Family: Combretaceae) and aerial part of *Eclipta alba* (Family: Asteraceae) for its phytochemical constituents and selected pharmacological activities (diuretic and antidiabetic). Diuretic medications are used to treat hypertension. Diabetes and hypertension are two common disease of geriatric patients and our aim was to explore medicinal plants of Sundarbans to find therapeutic of these diseases. The presence of carbohydrates, glycosides, reducing sugar, tannins, flavonoids, alkaloids, proteins, gum, steroids, saponin, and acidic chemicals was shown by phytochemical study of *L. racemosa* and *E. alba* showed the existence of tannin, saponin, flavonoid, gum, alkaloid, steroid, and terpenoid. Both plants extract was fractionated depending on polarity using *n*-hexane (non-polar), ethyl acetate (medium-polar) and water (polar). In the diuretic activity assay using Swiss Albino mice, none of the fractions of *L. racemosa* and *E. alba* showed diuretic activity but *n*-hexane 500 mg/kg of *E. alba* exhibited little diuretic activity compared to the standard frusemide. Swiss Albino mice were used to assess the oral glucose tolerance test (OGTT) in order to measure antidiabetic activity. In OGTT, water fraction 500 mg/kg of *L. racemosa* and *E. alba* showed blood glucose lowering activity compared to the standard Glibenclamide. We had performed *in silico* study among reported eight (08) compounds of *L. racemosa* against 1V4S (human glucokinase) protein. In comparison to classic glibenclamide (-8.5 kcal/mol), myricitrin had a good docking score of -8.4 kcal/mol. Based on these findings, we hypothesized that *L. racemosa* and *E. alba* could be a possible source of therapeutic leads for hypertension and hyperglycemia.

Keywords: *Lumnitzera racemosa*, *Eclipta alba*, Diuretic activity, Antidiabetic activity, Molecular Docking Study.

INTRODUCTION

Medicinal plants are widely used all over the world for production of both traditional medicine and modern drugs as well as development of new drugs. According to estimates from the World Health Organization (WHO), 80% of people in developing nations, get their primary medical care from plant-based medications traditional medicines. The need for medicinal plants in Bangladesh for the manufacture of traditional medicines (ayurvedic, unani and homeopathic) is rising day by day. Bangladesh has a rich flora of medicinal plants with a number of important active principles that have been used traditionally against a wide variety of diseases. Due to unwanted side-effects of synthetic and semisynthetic drugs, attention has been focused on utilizing natural medicine. Many people of our country have been suffering from diabetes and hypertension. Drugs called diuretics are used to treat hypertension. People used allopathic medicines for their treatment of diabetes and high blood pressure and these medicines show many adverse effects. Our target will explore medicinal plants of Sundarban to

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find new herbal medicine for the management of hypertension and diabetes

Mangrove species *Lumnitzera racemosa* (Family: Combretaceae) is widely distributed in tropical and subtropical regions. *L. racemosa* is a small tree or evergreen shrub that can reach a height of 8 meters. It has numerous spreading branches that are covered with twigs that are red, reddish-brown, or greyish-black, and the bark is brown to greyish-black. Flowering season varies from region to region. Qualitative studies reveal that the extracts of leaves, stems, and bark contain sugars, terpenoids, tannins, steroids, phenols, flavonoids, glycosides, coumarins, alkaloids, essential oils, anthraquinones, and saponins. [1]. Previous studies reported that *L. racemosa* has antibacterial, antifungal, antifungal, antidiarrheal, hepatoprotective and cytotoxic activities [2].

Asteraceae family member *Eclipta alba* is referred to as Bhringaraja and False daisy. It is a little annual herb that is frequently utilized for a range of therapeutic uses. It has numerous branches and can be horizontal or vertical. The majority of the second branches emerge from massive, up to 7 mm long, grey roots. Due to repressed white hair, prominent nodes, and red color, the stem is round or flat and rough. Leaves: oblong, lanceolate, sub-entire, strigose, 2.0–6.2 cm long, 1.5–1.9 cm wide, opposite, sessile to sub-sessile, acute to subacute, and hair oppressed on both faces. All year long, flowers bloom. *E. alba* has analgesic, hepatoprotective, antidiabetic, hair growth promoting, antioxidant, cardiovascular, anti-microbial, anti-epilepsy, anticancer, antiulcer, anti-venom, neuropharmacological and antihyperlipidemic properties [3]. Medicinal plants from different regions possess different biological activities.

The purpose of this experiment was to explore the antidiabetic and diuretic properties of two commonly used medicinal plants from the Sundarbans, *L. racemosa* and *E. alba*, using a molecular docking analysis of isolated chemicals to determine their mechanism of action. The identification of a novel biological activity in these plants could serve as a foundation for the creation of important medicines with improved pharmacological characteristics (safety and efficacy). These studies enable us to pinpoint the active ingredients that give medical effects and assist raise public awareness of the need of cultivating and protecting these kinds of medicinal plants. In the perspective of developing countries like Bangladesh, research on indigenous medicinal plants has also great potential of contributing substantially to the economy of the country. To achieve sustainable development goals (SDGs) we need safe, effective, quality and affordable essential medicines for all. The government is working hard to bring back traditional cultures and wants to promote domestic medicine over imported medications since both are accessible and affordable, which eventually strengthens the national economy.

MATERIALS AND METHODS

Plant Material and Extraction

The aerial portions of *E. alba* and the leaves of *L. racemosa* were gathered from the Sundarbans. Adulteration of any kind was forbidden during collection. Researchers from Khulna University's Forestry and Wood Technology Faculty and the Bangladesh National Herbarium in Mirpur, Dhaka, Bangladesh, identified the plants. *E. alba*'s voucher specimen number is 46081 DACB. The harvested plant parts were chopped into tiny pieces and allow to dry for 21 days away from the sun. Following the final milling of the dried plant materials, 500 g of it were extracted using a 15-day maceration technique and 1200 mL of 80% ethanol. A filter removed the extracts. Using a rotary evaporator, the solvents were removed at room temperature. Water (polar), ethylacetate (medium polar), and n-hexane (non-polar) were utilized to fractionate the ethanolic extract. First the extract was mixed with 500 mL water and added 500 mL n-hexane with it. Then the mixture was taken in a separating funnel and shaken and stand for 30 minutes. n-hexane fraction was separated. Remaining water fraction was mixed with

500 mL ethylacetate and again taken in separating funnel and water and ethylacetate fraction were separated. A preliminary screening for phytochemical and pharmacological effects was performed on the crude extracts.

Animals

For the tests, Swiss-Albino mice of both sexes, aged 12–14 weeks and weighing 25–30gram body weight, were gathered from the animal resources department of the International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B). The animals were fed standard meals (ICDDR, B prepared) and housed in normal polypropylene cages. Prior conducting the experiment, the animals were allowed to acclimate for a total of 14 days at the animal house located in the Pharmacy Discipline of Khulna University. Standard laboratory settings included a relative humidity of 55–60%, a room temperature of 25–20 C, and a 12-hour light–dark cycle.

Chemicals

We procured sodium nitropruside and sodium molibdate from Merck in Germany. We purchased sodium phosphate, sulphanilamide, and N 1-naphthyl ethylenediamine dihydrochloride from BDH, England, along with ethylene diamine tetraacetic acid (EDTA). Sigma-Aldrich (USA) provided the following chemicals: sodium hydroxide, hydrochloric acid, ferric chloride, potassium dichromate, sulfuric acid, sodium nitroprusside, n-hexane, ethyl acetate, and ethanol. Frusemide sodium and Glibenclamide, two common medications, were obtained from Beximco Pharmaceuticals Ltd. in Dhaka, Bangladesh.

Phytochemical tests

A preliminary phytochemical screening was performed on the *L. racemosa* and *E. alba* extract in order to identify the main functional groups [4]. Each test involved taking a 2% (w/v) solution of the extracts in ethanol. The various chemical groups in the extract were identified using the following techniques. Subsequently, the extract was employed for pharmacological examination.

Tests for Reducing Sugar

Benedict's test requires filling a test tube with 0.5 mL of the plant material's ethanol extract. The test tube is filled with 5 mL of Benedict's solution, brought to a boil for 5 minutes, and then allowed to cool naturally. There is reducing sugar present when a red cuprous oxide precipitate forms.

Fehling's Test (Standard Test): After boiling for a few minutes, 1 mL of a mixture of equal volumes of Fehling's solutions A and B is added to 2 mL of an aqueous extract of the plant material. When reducing sugar was present, a red or brick red precipitate happened.

Test for Combined Reducing Sugar: 1 mL of a plant extract in water is heated for 5 minutes together with 2 mL of diluted hydrochloric acid. The mixture is then cooled and neutralized with sodium hydroxide solution.

Tests for Tannins

Test for Ferric Chloride: Five milliliter solutions of every extract are placed in separate test tubes. Next, a 1 mL solution containing 5% ferric chloride is added. Presence of tannins is proven by a greenish-black precipitate.

Test for potassium dichromate: Five milliliter volumes of each extract are placed in separate test tubes. Next, a 10% potassium dichromate solution (one milliliter) is added. Yellow precipitate indicates the existence of tannins.

Test for Flavonoids

An ethanolic extract of the plant materials is combined with a small amount of strong hydrochloric acid in a few drops. The presence of flavonoids is indicated by the rapid development of a red tint.

Test for Saponins

Each extract is diluted to a volume of 20 mL with distilled water, and the mixture is shaken in a graduated cylinder for a period of 15 minutes. The existence of saponins is indicated by the formation of a 1 cm layer of foam.

Test for Gums

After taking 5 mL solutions of each extract, add

sulfuric acid and Molish reagent. When two liquids combine, a reddish-violet ring is formed, signifying the presence of carbohydrates and gum.

Test for Steroids

Libermann-Burchard test: Add 2 mL of Libermann-Burchard reagent to a 1 mL solution of ethanolic extract. The presence of steroids is indicated by a reddish-purple hue.

Test for sulfuric acid: 1 milliliter of a chloroform extract solution is obtained, and 1 milliliter of sulfuric acid is added. Steroids are indicated by the color red.

Test for Alkaloids

Mayer's test: It involves filling a test tube with 0.2 mL of diluted hydrochloric acid and 2 mL of the extract solution. Mayer's reagent (1 mL) is then added. The presence of alkaloids is shown by the formation of a yellow precipitate.

Dragendroff's test: It involves filling a test tube with 0.2 mL of diluted hydrochloric acid and 2 mL of each extract solution. Next, add 1 milliliter of Dragendroff's reagent. Alkaloids are indicated by an orange-brown precipitate.

Wagner's test: It involves filling a test tube with 0.2 mL of diluted hydrochloric acid and 2 mL of each extract solution. Wagner's reagent (iodine solution) is then added in a volume of 1 mL. The occurrence of alkaloids can be seen by a reddish-brown precipitate.

Hager's test: In a test tube, 2 mL of each extract solution and 0.2 mL of diluted hydrochloric acid are added. Hager's reagent, or 1 mL of picric acid solution, is then added. Precipitate with a yellowish tint suggests the presence of alkaloids.

Test for Terpenoids

2 mL sample of the ethanolic extract solution was heated for about 2 minutes after 2 mL of concentrated H2SO4 was added. The presence of terpenoids was suggested by a grayish appearance.

Test for Glycosides

Little amount of 10% (w/v) NaOH was added to an aliquot containing 3 mL of the aqueous extract solution. Next, a small amount of recently made 10% (w/v) sodium nitroprusside was added to the combination solution. The lack of glycosides was demonstrated by the formation of no blue, pink, or red colour.

Oral glucose tolerance tests (OGTT) for Antidiabetic Assay

Both plants' OGTT were conducted using a slightly modified version of the method Joy and Kuttan, 1999 had previously published [5. 6]. The mice were chosen by chance and placed into six (06) groups, labeled group-I, group-II and group-III, group-IV, group V, and group VI, with five (05) mice in each group. The experimental animals were maintained in a fasting state (having no food or drink except water for at least 10 hours but not greater than 16 hours). Every group was given a certain treatment, such as the test sample, the standard, and the control. The doses of the test sample, reference, and control drugs were adjusted to comply with precise weight values of each mouse. Every group was given a specific therapy. Each mouse was accurately weighed and the dosages of the test and control materials were adjusted before commencing any treatment. Glibenclamide (10 mg/kg bogy weight), control (1% tween-80 solution in water) and test sample (250 mg/kg and 500 mg/kg) were given orally via feeding needle at zero-hour doses of. All groups received treatment with a 10% glucose solution (2 gm/kg body weight) after 30 minutes. By taking blood samples from the tail vein approximately 30, 60, 90, 120, and 150 minutes after the glucose was administered, blood glucose loading was ascertained. Lastly, a glucometer was used to measure the blood glucose level [5, 6]. The unit of measurement for blood glucose was mmol/L. At 0.05, the threshold for statistical significance was established.

Diuretic Activity Assay

Both plants' diuretic activity was assessed in compliance with the guidelines provided by Mekonnen *et al.* (2010) and Mamun *et al.* (2003) [7, 8]. Using a random selection process, the mice were split up into six groups (group I,

group II, group III, group IV, group V, and group VI), each with five (05) mice. Prior the experiment, all the groups were given an 18-hour fast and water deprivation. As a control, animals in group I were given normal saline (15 mL/kg, p.o.), while animals in group II were given the conventional medication furosemide (5 mg/kg, p.o.) in normal saline. The test groups, which were groups III, IV, V, and VI, were given n-hexane at doses of 250 and 500 mg/kg along with a water fraction in typical saline. The animals were put in metabolic cages that were specifically made to segregate urine and feces as soon as they were dosed (5 per cage; ugo basile®, Italy). The animals were kept in their cages at (25.0±0.5) °C for the duration of the experiment, and food and water were not given for six hours after the mice were placed in them. During the 6-hour treatment period, the urinary output (Vo) was obtained every hour, and the urine was then held at -20 °C for additional examination. To compare the extracts' effects on urine excretion, the following parameters were computed. The ratio of total urination to total liquid delivered was used to compute urinary excretion, which was independent of animal weight (Formula-I). A drug's diuretic activity at a particular dose was measured using the ratio of urine excretion in the test group to urine excretion in the control group (Formula-II). The extract's diuretic action was compared to the standard medication in the test group (Formula-III) in order to determine its diuretic activity. Cl^- was determined titrimetrically, and the electrolyte content (Na^+ and K^+) of the urine sample was checked with a flame photometer. Using the proper techniques, pH, conductivity, and density were also ascertained.

$$\text{Urinary excretion} = \frac{\text{Total urinary output } (V_0)}{\text{Total liquid administered } (V_i)} \times 100 \ldots\ldots \text{(I)}$$

$$\text{Diuretic action} = \frac{\text{Urinary excretion of treatment groups}}{\text{Urinary excretion of control group}} \ldots\ldots\ldots \text{(II)}$$

$$\text{Diuretic activity} = \frac{\text{Diuretic action of test groups}}{\text{Diuretic action of standard group}} \ldots\ldots\ldots\ldots \text{(III)}$$

***In silico* Molecular Docking Study**

The "Vina Wizard" program in PyRx - Python Prescription 0.8 was used to execute molecular docking investigation between the receptors and the ligands (separately) [9]. First, the receptors and the ligands (independently) were put into the PyRx program with the proper compound—that is, macromolecule or ligand—declaration. Two distinct, optimized cube-sized boxes with precise measurements that line up with the receptors' main active site residues. PyMOL then paired the upgraded receptor with the docked ligands (separately). To ensure adequate visualization, the combined structures were seen using Discovery Studio. Images were saved in the optimal positions after the ligand interactions with amino acids were seen.

Arrangement of protein molecules

The human glucokinase protein model, PDB ID 1V4S, was chosen and obtained from the protein bank (https://www.rcsb.org/) in order to conduct the molecular docking investigation. The Discovery studio 2020 client then processed the obtained protein 3D structures to eliminate all associated ligands and water molecules in order to prepare the environment for subsequently interacting with the chosen ligand molecules [10].

Setting up ligands

Glibenclamide (CID: 3488), a typical drug used in biological tests, has 3D structures that may be obtained from PubChem (https://pubchem.ncbi.nih.gov/). We choose eight (08) isolated chemicals from *L. racemosa* that have been reported [11]. Furthermore, we retrieved several compounds that were reported to be present in the *L. racemosa* from the same website, including methyl gallate (4) (CID: 7428), 3,4,3'-tri-O-methylellagic acid (3) (CID: 5281860), myricitrin (5) (CID: 5281673), stigmasterol (6) (CID: 5280794), kaempferol (7) (CID: 5280863), isoguaiacin (8) (CID: 10314441), and others. Chem3D was used to draw the other two chemicals, racelactone (1) and botulin (2). PyRx analyzed those in order to minimize energy use.

Site specific binding and visualization

In order to allow all of the chosen protein molecules to be available for binding with the conducted ligands, we made the decision to use the blind docking approach. PyRx and auto dock vina 4.2 were used for these procedures, and the grid dimensions for the 1V4S protein were x: y: z = 58.7568:76.6570:65.3039 [10].

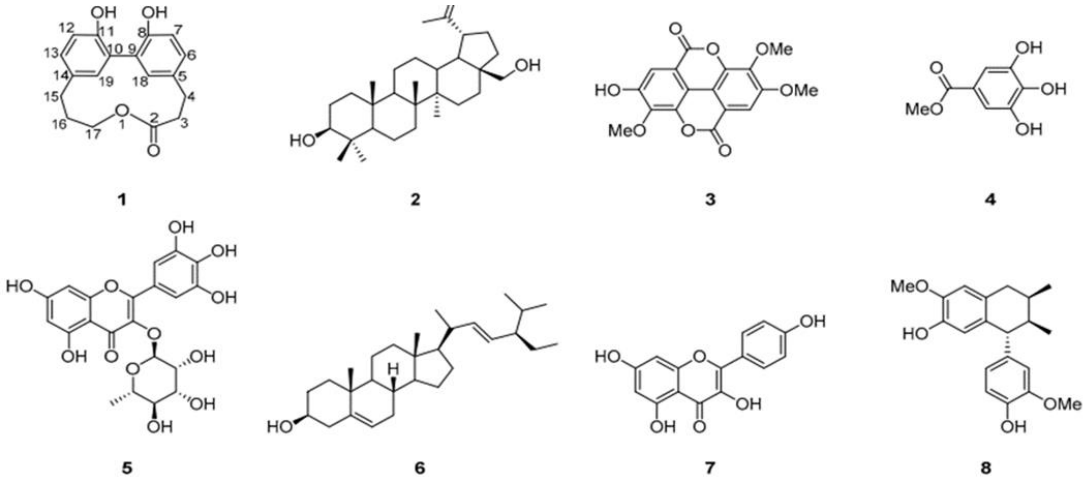


Figure 1: Reported compounds isolated from *L. racemose*

Analytical statistics

Using Excel and Prism, the Student's t-test was applied to identify significant differences between the test group and the control group.

RESULTS AND DISCUSSION

Results

Numerous secondary metabolites, including reducing sugars, flavonoids, phenolic compounds, tannins, proteins, glycosides, alkaloids, saponins, steroids, terpenoids, and acidic compounds, were found in the phytochemical analysis of the *L. racemosa* and *E. alba* extract. These metabolites may be responsible for some of the plant's therapeutic qualities (Table 1).

Table 1: Outcome of *L. racemosa* and *E. alba* extract Chemical Group Tests

Constituent	*L. racemosa*	*E. alba*
Phenolic Compound	+	+
Flavonoids	+	+
Carbohydrates	+	-
Reducing Sugar	+	-
Tannins	+	+
Saponin	-	+
Glycoside	+	-
Gums	-	+
Alkaloids	+	+
Protein	+	-
Acidic Compounds	+	+

Antidiabetic activity assay

The antidiabetic activity of the different fractions of *L. racemosa* and *E. alba* extract were examined using OGTT. Blood sugar levels declined in a dose-dependent way in the water fractions of the *L. racemosa* extract. 5.20 and 4.30

mMol/L at the dose of 500 mg/kg body weight in contrast to the reference drug Glibenclamide 5.06 and 4.1 mMol/L after 90 and 150 min (Table 2).

The amount of glucose in the blood dropped in a dose-dependent way in the water fractions of the *E. alba* extract. 5.32 and 5.46 mMol/L at the dose of 250 mg/kg body weight in contrast to the reference drug Glibenclamide 5.44 and 4.58 mMol/L after 90 and 150 min (Table 3).

Table 2: Oral Sugar Tolerance test result of *L. racemosa* extract

Administered dose & Test Groups	Mice Number	Mice Weight(gm)	Blood Sugar Level (mmol/L)			
			At Fasting State (At 0 min)	After the Administration of Glucose		
				At 30 min	At 90 min	At 150 min
Control	1	27	5.4	13.6	7.6	6.6
	2	25	6.4	16.0	8.2	5.8
	3	28	5.3	11.8	7.3	6.8
	4	26	5.5	12.3	8.6	6.2
	5	27	6.1	14.1	8.0	5.9
	Average		**5.74**	**13.56**	**7.94**	**6.26**
Positive control Glibenclamide (5 mg/kg)	1	26	4.7	10.8	6.1	5.2
	2	25	4.2	13.7	5.6	4.5
	3	25	5.6	9.4	3.8	3.0
	4	27	5.9	11.2	4.9	3.4
	5	27	4.8	10.2	6.3	4.1
	Average		**5.04**	**11.06**	**5.06**	**4.1**
***n*-hexane fraction of *L. racemosa* (250 mg/kg)**	1	25	5.3	10.1	5.6	5.2
	2	25	6.0	9.1	6.9	5.8
	3	27	5.7	10.5	5.8	4.9
	4	26	4.9	12.1	6.5	5.9
	5	28	5.2	11.5	6.2	6.0
	Average		**5.42**	**10.74**	**6.25**	**5.56**
***n*-hexane fraction of *L. racemosa* (500 mg/kg)**	1	25	4.8	10.0	6.5	4.6
	2	28	6.4	11.7	7.6	6.2
	3	26	5.9	12.1	7.9	6.7
	4	25	6.1	10.3	5.4	4.9
	5	27	5.2	11.2	6.3	5.9
	Average		**5.68**	**11.06**	**6.74**	**5.66**
H_2O fraction of *L. racemosa* (250 mg/kg)	1	26	5.7	10.3	6.5	5.7
	2	26	6.3	11.1	7.2	6.2
	3	27	5.9	12.5	7.9	6.9
	4	25	4.9	10.5	6.8	5.9
	5	28	6.2	12.2	5.9	4.9
	Average		**5.8**	**11.32**	**6.86**	**5.92**
H_2O fraction of *L. racemosa* (500 mg/kg	1	26	5.9	10.5	5.0	4.5
	2	28	6.7	12.1	5.4	4.2
	3	25	4.9	10.2	5.1	4.4
	4	27	5.2	11.5	5.2	4.3
	5	25	4.2	11.6	5.3	4.1
	Average		**5.38**	**11.18**	**5.20**	**4.30**

Table 3: Oral Sugar Tolerance Test result of *E. alba* extract

Administered dose & Test Group	Mice Number	Weight (gm) of mice	Blood Glucose Level (mmol/L)			
			At Fasting State (At 0 min)	After the Administration of Glucose		
				At 30 min	At 90 min	At 150 min
Control	1	28	5.5	12.5	8.2	5.9
	2	29	6.3	15.0	7.5	6.7
	3	28	5.5	12.9	8.5	5.8
	4	29	5.7	13.5	7.9	6.3
	5	27	6.3	14.7	8.3	6.2
	Average		**5.86**	**13.72**	**8.08**	**6.18**
Positive control Glibenclamide (5 mg/kg)	1	28	5.0	11.1	5.9	4.8
	2	29	4.7	12.2	5.5	4.6
	3	27	5.3	10.9	4.8	4.3
	4	27	5.5	11.5	5.2	4.5
	5	29	4.9	11.4	5.8	4.7
	Average		**5.08**	**11.42**	**5.44**	**4.58**
***n*-hexane fraction of *E. alba* (250 mg/kg)**	1	29	5.5	12.9	8.4	7.4
	2	28	5.6	13.2	8.8	6.9
	3	27	5.4	13.6	8.9	6.8
	4	28	5.9	13.4	9.2	7.2
	5	28	5.7	13.8	9.3	7.0
	Average		**5.62**	**13.38**	**8.92**	**7.06**
***n*-hexane fraction of *E. alba* (500 mg/kg)**	1	29	5.8	12.1	7.8	7.5
	2	28	5.9	11.9	7.7	7.9
	3	29	5.7	12.4	7.9	7.7
	4	29	5.8	12.6	8.4	7.8
	5	27	5.6	12.3	8.3	7.5
	Average		**5.76**	**12.26**	**8.02**	**7.68**
H_2O fraction of *E. alba* (250 mg/kg)	1	29	5.5	11.1	5.4	5.3
	2	29	6.1	11.4	5.3	5.4
	3	27	5.8	11.9	4.9	5.5
	4	29	5.9	11.5	5.1	5.2
	5	28	6.1	12.1	5.9	5.9
	Average		**5.88**	**11.60**	**5.32**	**5.46**
H_2O fraction of *E. alba* (500 mg/kg	1	29	6.1	10.6	5.6	5.6
	2	28	5.7	11.1	5.7	5.8
	3	28	5.9	10.8	5.9	5.4
	4	27	5.1	11.7	5.9	5.3
	5	29	5.2	11.9	5.5	5.8
	Average		**5.6**	**11.22**	**5.72**	**5.58**

Diuretic Activity Assay

Different fractions of *L. racemosa* and *E. alba* extract failed to produce an increase in urine volume elimination when compared to the normal dose of Frusemide (5 mg/kg-bw) in the mice used to evaluate its diuretic activity (Table 4, 5, 10 & 11). Only n-hexane fraction of *E. alba* showed mild increase in urine volume but lower than to the standard Frusemide 5 mg/kg-bw. However, electrolyte

analysis of urine samples the *L. racemosa* and *E. alba* extract showed a considerable concentration of potassium, sodium, and chloride ion in the sample that was comparable to control and standard (Table 6, 7, 8, 9, 12, 13, 14, 15).

Table 4: Urinary output of different fractions of *L. racemosa*

Groups	Urine volume (V_O) mL						Urinary excretion, (V_O/ V_i) × 100					
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Group-I (Negative Control)	0.5	1.6	2.1	2.5	3	3.6	4.1	13.33	17.5	20.83	25	30
Group-II Frusemide (5 mg/kg)	3.9	7.1	9	10	11.3	13.7	32.5	59.17	75	87.5	94.17	114.7
Group-III (*n*-hex. 250 mg/kg)	0.5	1	2	2.25	3	3.25	4.17	8.33	16.67	18.75	25	27.08
Group-IV (*n*-hex. 500 mg/kg)	0.5	1.75	2	3	3.75	4	4.16	14.58	16.67	25	31.25	33.33
Group-V (Water 250 mg/kg)	0	.25	1.75	2	2	2.25	0	2.08	14.58	16.67	16.67	18.75
Group-VI (Water 500 mg/kg)	0	1	2.25	1.75	2	2.5	0	8.33	18.75	14.58	16.67	20.83

Table 5: Diuretic action and activity of different fractions of *L. racemosa*

Groups	Diuretic action (U_{ET}/ U_{EC})						Diuretic activity (D_{AT}/ D_{AU})					
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Group-I (Negative Control)	-	-	-	-	-	-	-	-	-	-	-	-
Group-II Frusemide (5 mg/kg)	7.8	4.43	4.28	4.2	3.77	3.81	-	-	-	-	-	-
Group-III (*n*-hexane 250 mg/kg)	1	0.63	0.95	0.9	1	0.9	0.12	0.14	0..22	0.26	0.27	0.24
Group-IV (*n*-hexane 500 mg/kg)	1	1.09	0.95	1.2	1.25	1.11	0.13	0.27	0.22	0.3	0.33	0..29
Group-V (Water 250 mg/kg)	0	0.16	0.83	0.8	0.67	0.63	0	0.04	0.19	0.2	0.2	0.18
Group-VI (Water 500 mg/kg)	0	0.63	1.07	0.7	0.67	0.69	0	0.14	0.25	0.18	0.17	0.18

Table 6: Concentration of Na+ in different fractions of *L. racemosa*

Groups	Flame Intensity (FI) vs [Na^+]				Concentration of Na^+ (mEq/L) ± SEM
	Replication 1		Replication 2		
	Flame Intensity	[Na^+] (mEq/L)	Flame Intensity	[Na^+] (mEq/L)	
Control (Normal Saline)	0.60	181.90	0.60	181.90	181.90 ± 0
Frusemide (5 mg/kg)	0.90	279.91	0.91	283.18	281.55 ± 1.64
Fr. *n*-hexane (250 mg/kg)	0.68	208.04	0.72	221.18	214.61 ± 6.57
Fr. *n*-hexane (500 mg/kg)	0.74	227.64	0.80	247.24	237.44 ± 9.8
Fr. H_2O (250 mg/kg)	0.62	188.44	0.66	201.51	194.97± .653
Fr. H_2O (500 mg/kg)	0.66	201.51	0.68	208.04	204.77± 3.26

Table 7: Concentration of K^+ in different fractions of *L. racemosa*

Groups	Flame Intensity (FI) vs [K^+]				Concentration of K^+ (mEq/L) ± SEM
	Replication 1		Replication 2		
	Flame Intensity	[K^+] (mEq/L)	Flame Intensity	[K^+] (mEq/L)	
Control (Normal Saline)	0.35	19.65	0.35	19.65	19.65 ± 0
Frusemide (5 mg/kg)	0.55	42.79	0.56	43.95	43.37 ± 0.58
Fr. *n*-hexane (250 mg/kg)	0.45	31.21	0.46	32.37	31.79 ± 0.58
Fr. *n*-hexane (500 mg/kg)	0.48	34.68	0.46	32.37	33.52 ± 1.15
Fr. H_2O (250 mg/kg)	0.40	25.43	0.42	27.73	26.58± 1.15
Fr. H_2O (500 mg/kg)	0.42	27.73	0.45	31.21	29.47 ± 1.47

Table 8: Concentration of Cl^- in different fractions of *L. racemosa*

Groups	Replication 1		Replication 2		Average conc. of Cl^- (mEq/L) ± SEM
	Volume of Titre (mL)	[Cl^-] (mEq/L)	Volume of Titre (mL)	[Cl^-] (mEq/L)	
Control (Normal Saline)	0.086	90	0.086	90	90 ± 0
Frusemide (5 mg/kg)	0.097	117.5	0.099	122.5	120 ± 2.5
Fr. *n*-hexane (250 mg/kg)	0.089	104	0.092	105	104.5 ± 0.5
Fr. *n*-hexane (500 mg/kg)	0.092	111.5	0.094	114.5	113 ± 1.5
Fr. H_2O (250 mg/kg)	0.088	95	0.088	95	95 ± 0
Fr. H_2O (500 mg/kg)	0.090	100	0.093	101	100.5 ± 0.5

It is generally accepted that the Carbonic Anhydrase Inhibition (CAI) Index, K^+/Na^+ (Kaliuretic Index), Na^+/K^+ (Natriuretic Index), and Saluretic Index have a significant role in interpreting the mode of action of diuretic medications. The above parameters were also estimated as following-

Table 9: Effect of different treatment groups on Saluretic, Natriuretic, Kaluretic and CAI index of different fractions of *L. racemosa*

Groups	Cumulative ion Concentrations (mEq/L/6h)			Saluretic Index			Na^+ / K^+	K^+ / Na^+	CAI = {Cl^-/ (Na^++ K^+)}	Diuretic Index (DI)
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-				
Control (Normal Saline)	181.90	19.65	90	0	0	0	9.26	0.11	0.45	
Frusemide (5 mg/kg)	281.55	43.37	120	1.55	2.21	1.33	6.49	0.15	0.37	3.8
Fr. *n*-hexane (250 mg/kg)	214.61	31.79	104.5	1.14	1.58	1.15	6.75	0.14	0.42	0.90
Fr. *n*-hexane (500 mg/kg)	237.44	33.52	113	1.25	1.76	1.23	6.64	0.14	0.41	1.11
Fr. H_2O (250 mg/kg)	194.97	26.58	95	1.03	1.29	1.05	7.08	0.13	0.45	0.63
Fr. H_2O (500 mg/kg)	204.77	29.47	100.5	1.12	1.41	1.11	6.94	0.16	0.44	0.69

Diuretic index (DI) = Test group urine volume / Control group urine volume

Table 10: Urinary output of different fractions of *E. alba*

Groups	Urine Volume (V_o)						Urinary Excretion (V_o/V_i) ×100					
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Group-I Control (0.9% NaCl)	1.5	1.7	2	2.1	2.2	2.2	12.5	14.17	16.7	17.5	18.3	18.3
Group-II Standard (Furosemide 5 mg/kg)	6.6	6.8	7.5	7.6	7.8	7.8	55	56.67	62.5	63.3	65	65
Group-V n-Hexane (250mg/kg)	4.2	4.3	4.4	4.5	4.6	4.6	35	35.83	36.7	37.5	38.3	38.3
Group-VI n-Hexane (500mg/kg)	5.7	5.9	6.2	6.4	6.5	6.5	47.5	49.17	51.7	53.3	54.2	54.2
Group-IX Water (250mg/kg)	2	2.2	2.4	2.5	2.5	2.6	16.7	18.3	20	20.83	20.83	21.7
Group-X Water (500mg/kg)	2	2.5	2.9	3	3	3.1	16.7	20.83	24.2	25	25	25.8

Table 11: Diuretic action and activity of different fractions of *E. alba*

Groups	Diuretic Action (U_{ET}/U_{EC})						Diuretic Activity (D_{AT}/D_{AU})					
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Group-I Control (0.9% NaCl)	-	-	-	-	-	-	-	-	-	-	-	-
Group-II Standard (Furosemide 5mg/kg)	4.4	3.9	3.74	3.62	3.55	3.55	-	-	-	-	-	-
Group-V n-Hexane (250mg/kg)	2.8	2.53	2.2	2.14	2.09	2.09	0.64	0.65	0.59	0.59	.59	.59
Group-VI n-Hexane (500mg/kg)	3.67	3.47	3.1	3.05	2.96	2.96	0.85	0.89	0.83	0.84	.83	.83
Group-IX Water (250mg/kg)	1.34	1.29	1.2	1.19	1.14	1.19	0.30	0.33	0.32	0.33	.32	.34
Group-X Water (500mg/kg)	1.34	1.47	1.44	1.43	1.37	1.41	0.30	0.38	0.39	0.4	.39	0.4

Table 12: Concentration of Na^+ in different fractions of *E. alba*

Groups	Flame Intensity (FI) vs [Na^+]				Concentration of Na^+ (mEq/L) ± SEM
	Replication 1		Replication 2		
	Flame Intensity	[Na^+] (mEq/L)	Flame Intensity	[Na^+] (mEq/L)	
Control (Normal Saline)	0.62	182.70	0.61	183.30	183.00 ± 0
Frusemide (5 mg/kg)	0.91	280.88	0.92	284.32	282.60 ± 1.72
Fr. *n*-hexane (250 mg/kg)	0.68	209.21	0.71	220.34	214.76 ± 5.57
Fr. *n*-hexane (500 mg/kg)	0.75	229.86	0.78	246.45	238.16 ± 8.3
Fr. H_2O (250 mg/kg)	0.60	185.23	0.62	195.26	190.25 ± 5.02
Fr. H_2O (500 mg/kg)	0.63	193.64	0.61	201.37	197.51 ± 3.87

Table 13: Concentration of K^+ in different fractions of *E. alba*

Groups	Flame Intensity (FI) vs [K^+]				Concentration of K^+ (mEq/L) ± SEM
	Replication 1		Replication 2		
	Flame Intensity	[K^+] (mEq/L)	Flame Intensity	[K^+] (mEq/L)	
Control (Normal Saline)	0.33	18.79	0.34	18.48	18.64 ± 0
Frusemide (5 mg/kg)	0.56	43.85	0.56	43.90	43.88 ± 0.03
Fr. *n*-hexane (250 mg/kg)	0.44	30.33	0.45	31.64	30.99 ± 0.16
Fr. *n*-hexane (500 mg/kg)	0.46	32.34	0.45	31.88	32.11 ± 0.23
Fr. H_2O (250 mg/kg)	0.41	25.98	0.43	28.65	27.32 ± 1.34
Fr. H_2O (500 mg/kg)	0.43	28.34	0.44	30.86	29.60 ± 1.26

Table 14: Concentration of Cl^- in different fractions of *E. alba*

Groups	Replication 1		Replication 2		Average conc. of Cl^- (mEq/L) ± SEM
	Volume of Titre (mL)	[Cl^-] (mEq/L)	Volume of Titre (mL)	[Cl^-] (mEq/L)	
Control (Normal Saline)	0.087	91	0.085	89	90 ± 0
Frusemide (5 mg/kg)	0.096	116.6	0.099	122.8	119.7 ± 3.1
Fr. *n*-hexane (250 mg/kg)	0.088	101.16	0.089	101.98	101.57 ± 0.41
Fr. *n*-hexane (500 mg/kg)	0.091	109.76	0.093	111.81	110.79 ± 1.03
Fr. H_2O (250 mg/kg)	0.087	93.22	0.086	90.87	92.05 ± 1.18
Fr. H_2O (500 mg/kg)	0.091	100.2	0.093	101.7	100.5 ± 0.75

Table 15: Effect of different treatment groups on Saluretic, Natriuretic, Kaluretic and CAI index of different fractions of *E. alba*

Groups	Cumulative ion Concentrations (mEq/L/6h)			Saluretic Index			Na^+ / K^+	K^+ / Na^+	CAI = {Cl^-/ (Na^++ K^+)}	Diuretic Index (DI)
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-				
Control (Normal Saline)	183	18.64	90	0	0	0	9.82	0.10	0.45	
Frusemide (5 mg/kg)	282.60	43.88	119.7	1.54	2.35	1.33	6.44	0.16	0.37	3.55
Fr. *n*-hexane (250 mg/kg)	214.76	30.99	101.57	1.17	1.66	1.13	6.93	0.14	0.41	2.10
Fr. *n*-hexane (500 mg/kg)	238.16	32.11	110.79	1.30	1.72	1.23	7.42	0.13	0.49	2.95
Fr. H_2O (250 mg/kg)	190.25	27.32	92.05	1.03	1.47	1.02	6.96	0.14	0.42	1.18
Fr. H_2O (500 mg/kg)	197.51	29.60	100.5	1.07	1.59	1.12	6.67	0.15	0.44	1.41

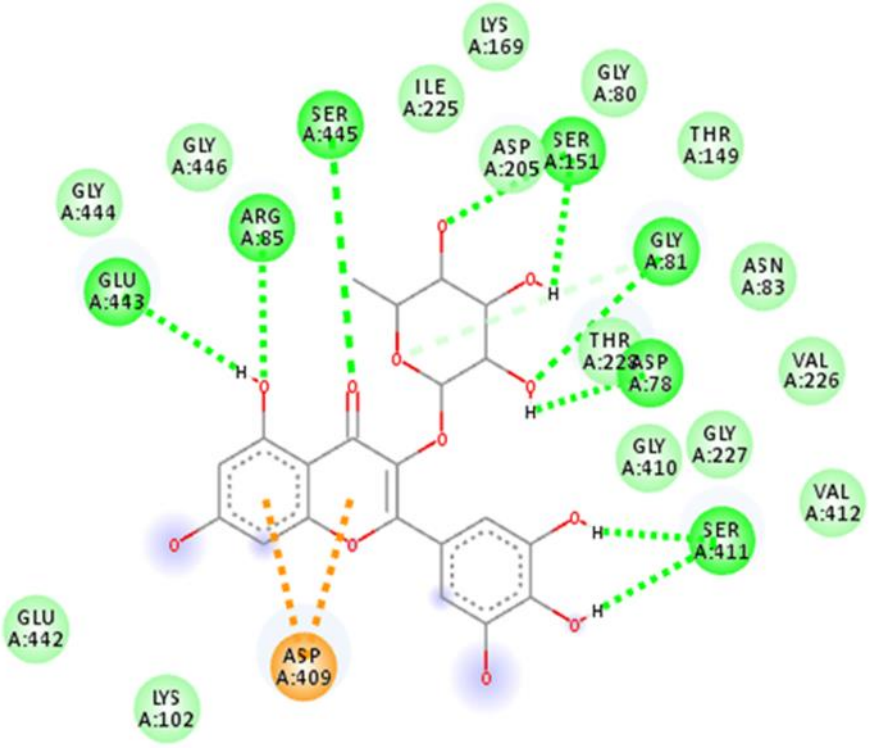
Diuretic index (DI) = Urine volume of test group/ Urine volume of control group

Molecular Docking Investigation

Molecular docking algorithms are frequently employed to forecast ligand energies and binding trends to proteins. The present study screened 08 compounds from *L. racemosa* against the diabetes-specific target protein Glucokinase (1V4S). According to the docking results, Myricitrin (**5**), Botulin (**2**), Kaempferol (**7**), and Racelactone (**1**) have the lowest docked binding energy compared to the standard Glibenclamide (Table 16).

Table 16: Docking score of reported eight (08) ligands from *L. racemosa* with 1V4S protein

Sl. No	Name of the ligands	Docking score (kcal/mol)
1	Racelactone (**1**)	-7.5
2	Botulin (**2**)	-7.9
3	3,4,3'-Tri-O-methylellagic acid (**3**)	-7.4
4	Methyl gallate (**4**)	-6.2
5	Myricitrin (**5**)	**-8.4**
6	Stigmasterol (**6**)	-7
7	Kaempferol (**7**)	-7.6
8	Isoguaiacin (**8**)	-7.3
9	Glibenclamide	**-8.5**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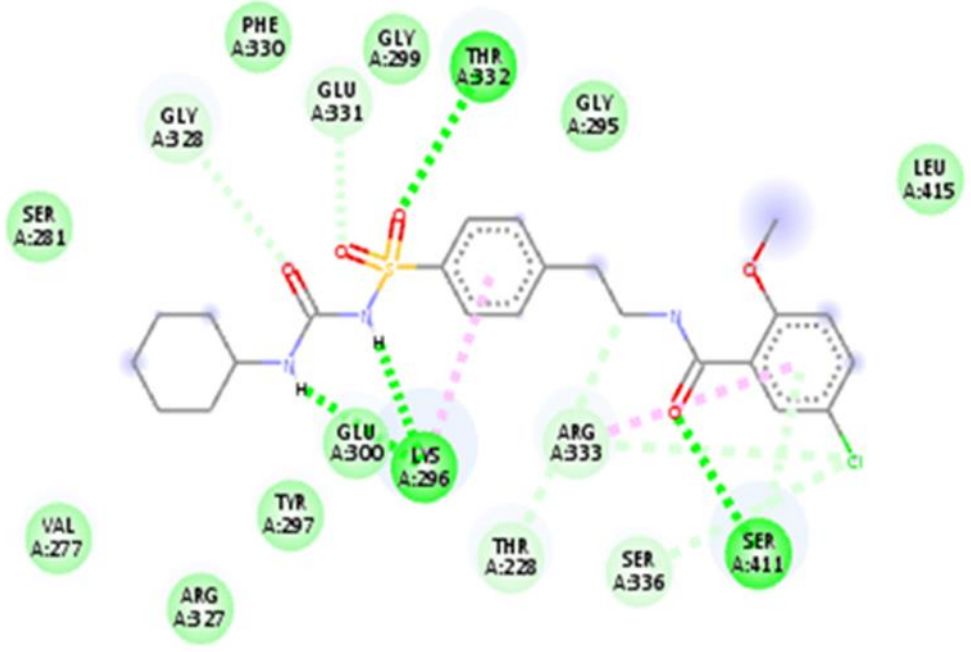
(A)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LYS A:296
ARG A:333
TYR A:297
VAL A:277
THR A:228
SER A:336
SER A:411
ARG A:327

(B)

Figure 2: 2D interactions of (A) Myricitrin and (B) Glibenclamide with 1V4S protein

Table 17: Ligand binding to 1V4S protein amino acids

Ligands	**Amino acid interactions**
Myricitrin	Asp78, Asp205, Asp409, Lys102, Lys169, Glu442, Glu443, Gly80, Gly81, Gly410, Gly227, Gly444, Gly446, Arg85, Ser151, Ser445, Ser411, Ile225, Thr149, Thr228, Asn83, Val226, Val412.
Glibenclamide	Ser281, Ser336, Ser411, Gly295, Gly299, Gly328, Phe330, Glu300, Glu331, Thr228, Thr332, Leu415, Arg327, Arg333, Lys296, Tyr297, Val277.

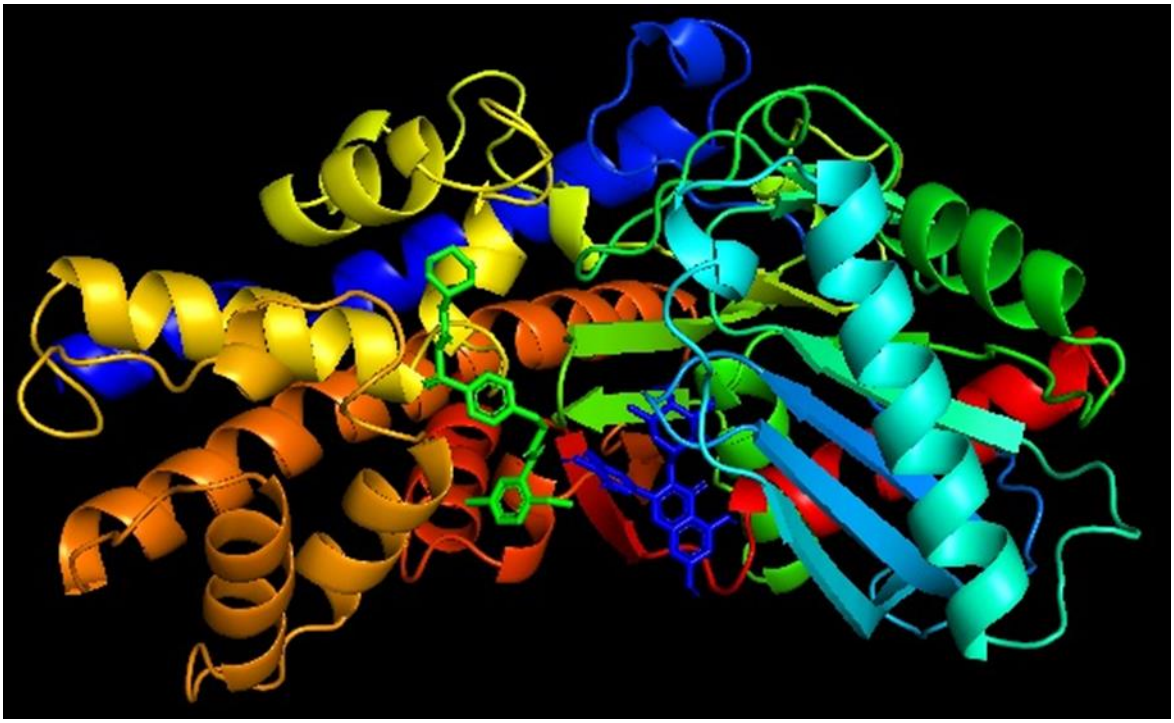

Figure 3: 3D interactions of Myricitrin (blue) and Glibenclamide (green) with 1V4S protein

DISCUSSION

Plants are blessings of mankind for its copious medicinal properties to defend against different diseases. Plants synthesize different secondary metabolites for its own protection from insects, microorganisms, diseases, and herbivorous mammals. These secondary metabolites perform momentous role on human body in various physiological disorders. According to WHO statistics, 80% of the global population either directly or indirectly uses medicinal plants to treat a variety of ailments. Preliminary phytochemical testing of the extracts of *L. racemosa* and *E. alba* suggested the presence of proteins, glycosides, alkaloids, terpenoids, flavonoids, phenolic compounds, tannins, and acidic compounds. The most

effective phytochemicals for medicinal use are flavonoids, alkaloids, tannins, and phenolic compounds. Polyphenolic products, including tannins, flavonoids, and phenolic acids, have already been proven to elicit a variety of biological reactions, including laxative, anthelmintic, antioxidant, and anti-inflammatory effects [12]. Terpenoids, flavonoids, and tannins are examples of phytochemicals that have analgesic properties [13, 14]. However, various phytochemicals existed in *L. racemosa* and *E. alba* extract helped us to perform different pharmacological tests.

The oral glucose tolerance test (OGTT) is the most reliable test for identifying borderline diabetes mellitus. The capability of the body to use glucose in the bloodstream is termed as glucose tolerance. As a result, the "glucose tolerance test" is a useful diagnostic tool for detecting diabetes mellitus, insulin resistance, diminished beta-cell activity, and occasionally acromegaly and reactive hypoglycemia [15]. Water fraction 500 mg/kg of *L. racemosa* extract gradually declines plasma glucose level in contrast to reference Glibenclamide. After 120 min, Water fraction of *L. racemosa* extract showed 4.58 mmol/L plasma sugar level at the dose of 500 mg/kg body weight compared to Glibenclamide 4.1 mmol/L. According to these results, Water fraction of *L. racemosa* extract exhibited hypoglycemic effect.

Diabetes is the ninth leading cause of death globally and one of the most common metabolic illnesses. Although there are reliable hypoglycemic agents for the control of diabetes, researchers are focusing upon multiple metabolic factors such as transporters, enzymes, and receptors in attempts to find a drug that is both more effective and less side effects. The enzyme glucokinase (GCK), which is mostly present in the liver and pancreatic beta cells, maintains blood glucose homeostasis [16]. One of the four hexokinase isozymes, glucokinase (GCK), converted glucose to glucose-6-phosphate in the hepatic and pancreatic cells. The rate at which glucose stimulates the synthesis of insulin is controlled by the perfect glucose sensor and rate-limiting enzyme GCK. Hence, the present *in silico* study is designed to determine the interaction between GCK and eight (08) reported compounds (ligands) of *L. racemosa*. In humans, pancreatic beta-cells and hepatoparenchymal cells create the majority of GCK [17]. In addition to aiding the liver in producing glycogen, GCK controls the pace at which insulin is produced by pancreatic cells in order to keep glucose homeostasis [18].

Molecular docking is a computer simulation approach that focuses on finding the ideal place of interaction between a chemical and a protein binding site. Using this method, the binding affinity is determined by predicting the ultimate orientation of the molecule within the binding site, which is chosen within the 3D coordinate space of the target. The significance and sensitivity of binding affinity values are determined by the largest negative value (greatest binding affinity or lowest binding energy), which represents the most valuable configuration of the complex formed when the associated ligand successfully binds with the target's active pockets [16]. According to binding affinity score Myricitrin (**5**) showed lowest binding score compared to standard Glibenclamide. Furthermore, by evaluating ligand binding modalities and orientation in the target protein 1V4S receptor pocket, the molecular docking model has been used to validate the anti-diabetic efficacy of Myricitrin (**5**). The ligand-receptor affinity was calculated using the binding free energy (G binding) value. The intermolecular energy, torsional free energy, and total internal energy were added to determine the binding free energy. As a result, the binding free energy was obtained and subtracted from the unbound system's energy. The optimal interaction site was found using the conformation with the lowest energy binding value.

Figure 2 showed the 2D interaction between glucokinase 1V4S protein with Myricitrin (**5**) and Glibenclamide. Compared to other ligands, Myricitrin (**5**) showed strong binding with glucokinase in terms of binding energy (-8.4 kcal/mol) compared to Glibenclamide (-8.5 kcal/mol). Myricitrin (**5**) formed

seven hydrogen bonding interactions with the amino acid residues of ARG85, ASP78, GLY81, SER151, SER411, GER445 and GLU443 (Figure 2). These kinds of 3D interactions were found in docking studies, which proved that particular ligand has a high affinity for the target protein (Figure 3).

Diuretics enhance the excretion of urine and optimize blood volume that finally regulate blood pressure. They primarily act on various nephron segments, increasing urine output. Diuretics may also accelerate the excretion of electrolytes. Diuretics are prescribed for treating a number of conditions, including kidney disease, influenza, water poisoning, hypertension, liver cirrhosis, and heart failure. A prior study found that a good diuretic activity is indicated by a diuretic index (DI) value greater than 1.50. On the other hand, the DI values between 0.72 and 0.99 and 1.00 and 1.50, respectively, show mild and moderate diuretic activity. There is no diuretic activity when the diuretic index value is less than 0.72 [19]. The extract displayed good diuretic activity as seen by the *n*-hexane fraction of *E. alba* extract, which showed DI 2.10 and 2.95 at the doses of 250 and 500 mg/kg while typical furosemide showed DI 3.55 (Table:15).

The plant extract's diuretic efficacy was dose- and time-dependent, suggesting that this impact is real, intrinsic, and potentially receptor-mediated [20]. For the management of hypertension, peripheral edema, ascites, and congestive heart failure, renal excretion of electrolytes is equally important as renal excretion of water [20]. The extract increased diuresis, which was evident in the electrolyte excretion as well. In a dose-dependent way, it markedly enhanced the excretion of urine electrolytes (Na^+, K^+, and Cl^-). Despite this, all fractions of the extract from *L. racemosa* and *E. alba* demonstrated higher K^+ excretion than the control. The ratio of Na^+/K^+ can be used to calculate the natriuretic activity (also known as the aldosterone secretory index) of the plant extract. Values higher than 2.0 suggest an effective natriuretic effect, whereas ratios higher than 10.0 indicate a potassium-sparing effect [21]. Between a score of six and seven, all fractions of the *L. racemosa* and *E. alba* extract showed strong natriuretic effects.

By inhibiting the Na^+-K^+-$2Cl^-$ symporter in the thick ascending limb of the loop of Henle, stimulating the production of renal prostaglandins, and inhibiting the carbonic anhydrase enzyme in the proximal convoluted tubule (PCT), loop diuretics increase the urinary flow rate and urinary excretion of Na^+, K^+, and Cl^- [22-24]. Since hypokalemia is one of the main side effects of loop and thiazide diuretics, oral K^+ supplements or potassium-sparing diuretics that lower urine K^+ excretion may be required [24-26]. The extract's ability to inhibit carbonic anhydrase is measured by the ratio of $Cl^-/ [Na^+ + K^+]$. It is possible to rule out carbonic anhydrase inhibition with values between 1.0 and 0.8. One can presume that there is enzyme inhibitory activity when the ratio decreases [21]. The carbonic anhydrase inhibitory indices of all fractions of *L. racemosa* and *E. alba* extract as well as normal Frusemide were less than 0.5 (Table 9 & 15). Accordingly, our study suggests that these plants may work as loop diuretics by inhibiting the carbonic anhydrase enzyme in the renal tubules, which raises urine pH.

CONCLUSION

Because medicines are essential to having a healthy population, which drives and sustains the economy, the significance of medicinal plants to the economy of developing nations like Bangladesh remains crucial and strategic. Maximum medicinal plants are abundantly indigenous to our country and we can use them at very cheap rates to produced different dosage forms that will boost our national economy. Pharmaceutical products based on medicinal plants strengthen the healthcare delivery system by providing the majority of the population with essential medications at reasonable costs. Moreover, not only medical benefits, implementation of this program will help boost revenue thereby contributing to wholesome national economy as well. Diuretics

decrease blood volume by enhancing urine volume and control blood pressure. Diuretic activity is directly related to kidney and other parts of urinary system. *L. racemosa* and *E. alba* have no traditional uses related to kidney and other parts of urinary system. So, in this experiment, both plants exhibited little increase in urine volume. Myricitrin (**5**) exhibited good docking score -8.4 kcal/mol against 1V4S protein that reflect good antidiabetic activity. So, Myricitrin (**5**) may be the lead compound for the future antidiabetic drug development. The results of this study will contribute to the increasing economic worth of medicinal plants, which developing nations must capitalize on to enhance their economic and healthcare systems.

Acknowledgement

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استكشاف الأنشطة المضادة لمرض السكر ومدرة للبول لنباتي Lumnitzera racemosa و Eclipta alba من خلال دراسة الالتحام الجزيئي

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ملخص

كان الهدف من الدراسة الحالية هو النظر في أوراق Lumnitzera Racemosa العائلة: Combretaceae والجزء الهوائي من Eclipta alba العائلة: (Asteraceaeلمكوناتها الكيميائية النباتية والأنشطة الدوائية المختارة (مدر للبول ومضاد لمرض السكر). تستخدم الأدوية المدرة للبول لعلاج ارتفاع ضغط الدم. يعد مرض السكري وارتفاع ضغط الدم من الأمراض الشائعة لدى مرضى الشيخوخة وكان هدفنا هو استكشاف النباتات الطبية في سوندربانس لإيجاد علاج لهذه الأمراض. أظهرت الدراسة الكيميائية النباتية لنبات L. Racemosa و E. alba وجود الكربوهيدرات والجليكوسيدات والسكر المختزل والعفص والفلافونويد والقلويدات والبروتينات والصمغ والمنشطات والسابونين والمواد الكيميائية الحمضية. الصمغ، قلويد، الستيرويد، وتيربينويد. تمت تجزئة مستخلص النباتين اعتمادًا على القطبية باستخدام ن-هكسان (غير قطبي)، وخلات الإيثيل (قطبي متوسط) والماء (قطبي). في اختبار نشاط مدر البول باستخدام فئران ألبينو سويسرية، لم يُظهر أي من أجزاء L. Racemosa وE. alba نشاطًا مدرًا للبول، لكن n-hexane 500 mg/kg من E. alba أظهر نشاطًا مدرًا للبول قليلًا مقارنة بالفروسيميد القياسي. تم استخدام الفئران البيضاء السويسرية لتقييم اختبار تحمل الجلوكوز عن طريق الفم ((OGTTمن أجل قياس النشاط المضاد لمرض السكر. في OGTT، أظهر جزء الماء 500 ملغم/كغم من L. Racemosa وE. alba نشاطًا لخفض نسبة الجلوكوز في الدم مقارنةً بـ Glibenclamide القياسي. لقد أجرينا دراسة سيليكو بين ثمانية (08) مركبات من L. Racemosa ضد بروتين 1) V4Sالجلوكوكيناز البشري). بالمقارنة مع جليبينكلاميد الكلاسيكي (-8.5 كيلو كالوري/مول)، كان للميريسيترين درجة إرساء جيدة تبلغ -8.4 كيلو كالوري/مول. بناءً على هذه النتائج، افترضنا أن L. Racemosa وE. alba يمكن أن يكونا مصدرًا محتملاً للعلاجات العلاجية لارتفاع ضغط الدم وارتفاع السكر في الدم.

الكلمات الدالة: Lumnitzera Racemosa، Eclipta alba، نشاط مدر للبول، نشاط مضاد لمرض السكر، دراسة الالتحام الجزيئي.

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Pharmacokinetic Evaluation of Niacin and Pterostilbene in Single and Multi-Doses in Healthy Subjects

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ABSTRACT

Background: The potential of natural antioxidants blends in metabolic syndromes and other ailments have been repeatedly investigated. Majority of studies are based on pharmacologic interactions and limited on their pharmacokinetic interactions. This study aimed to provide insight about pharmacokinetic interactions of niacin and pterostilbene upon concurrent administration and to quantify their blood concentrations in single and multiple doses.

Methodology: A randomized, open label, crossover design was followed to study pharmacokinetic interaction between niacin (NA) and pterostilbene (PT) in single- and multi-dose combinations in healthy volunteers. Subjects were administered with single and multiple doses (250mg/dose each) in individual and combinations for one week. Blood samples were collected and analyzed on newly developed HPLC-UV method for simultaneous quantification employing hexa-decyltrimethylammonium-bromide as ion pairing.

Results: Our method was found linear over applied concentration range (0.020-20µg mL^{-1}) and sensitive (lower quantification limits were 50 and 22ng mL^{-1} for NA and PT). Pharmacokinetic profiling (C_{max}, T_{max}, AUC, MRT, and $t_{1/2}$) of administered antioxidants showed no significant influence of analytes over one another in both single and multidose therapies. Comparing individual vs. combinations, statistically insignificant ($p>0.05$) variations were observed in plasma drug concentrations.

Conclusion: Findings of this study revealed the biocompatibility of test drugs proven by pharmacokinetic data and therefore can be used safely at their recommended doses in combined formulations.

Keywords: Pharmacokinetics; RP-HPLC-UV; carboxylic acids; natural antioxidants; area under curve.

1. INTRODUCTION

In current biomedical methodologies, the natural bioactive assisted therapies are gaining popularity worldwide and have been supported by strong epidemiological data in various chronic ailments, including cardiovascular disorders, neurodegenerative disorders, diabetes and cancer (1-4). Niacin (3-picolonic acid) an essential vitamin, also called nicotinic acid is a carboxylic acid containing organic compound, possessing cytoprotective (nicotinamide) effects such as in degenerative disorders, amoebic liver abscess and preventing brain cell damage after ischemic reperfusion (5). Niacin has also showed activity in restoring brain malondialdehyde and antioxidant enzymes thus help in maintaining the redox homeostasis (6). Various clinical studies have reported atherosclerotic preventive effects of niacin, improving cardiovascular outcome (7). It lipid

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modifying properties includes enhancing the high density lipoproteins (HDL) production, cholesterol efflux and reverse cholesterol transport. It enhances the clearance of triglyceride rich lipoprotein and decreases the production of VLDL (8, 9). Niacin is also reported as antioxidant, scavenging prooxidants radicals produced in liver fibrosis, ameliorating the hepatic physiology (10, 11).

Pterostilbene (PT) (trans-3, 5-dimethoxy-hydroxystilbene) is an analog of resveratrol possessing lipid lowering potential. It is phytoalexin (antimicrobial), present majorly in blue berries and heartwood of *Pterocrpus marsupium.* The substitution of two hydroxyl (OH) with methoxy-groups makes it more hydrophobic than resveratrol (Figure 2) (12, 13). Literature reported its anticancer activity by inducing apoptosis in cancerous cells, lipid modulation by enhancing physiological peroxisomes proliferated activated receptors activities and antidiabetic effect via enhancing sensitivity and release of insulin from pancreas. It has potentiating effects endogenous antioxidant system while suppressing reactive oxygen species (ROS) synthesis (14).

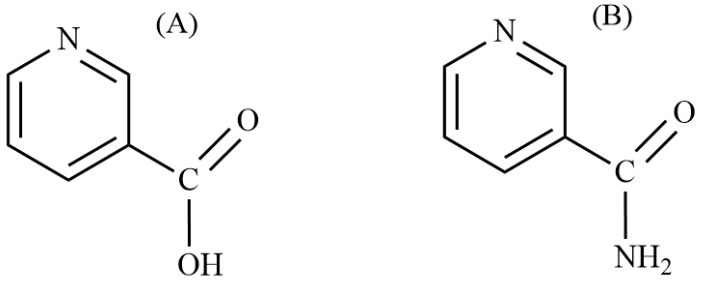

Figure 1: Chemical structures of niacin and nicotinamide

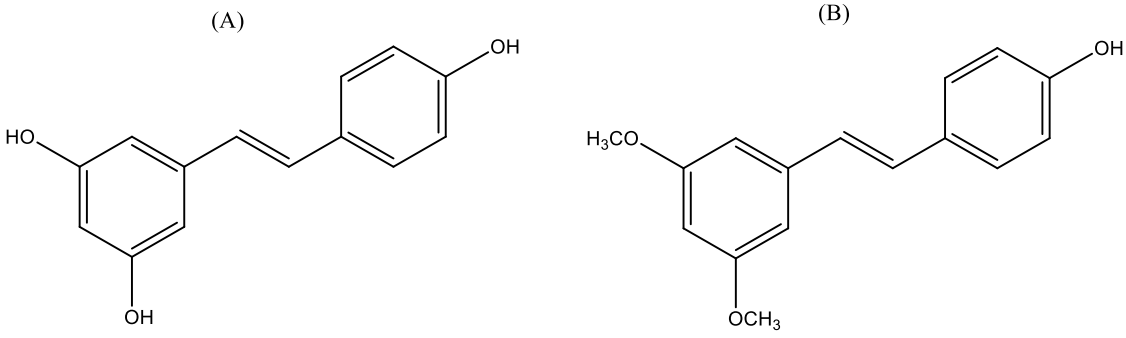

Figure 2: Chemical structures of resveratrol and pterostilbene with substituted methoxy groups

Numerous studies have been conducted on phytomedicine combination therapies with or without synthetic clinical medications for the treatment of biological deregulations in chronic conditions. The results confirmed the superiority of combination therapies in comparison to the individual high dose therapies, by providing synergistic effects (15-20). Niacin has been co-administered frequently with various lipid-modifying drugs including statins, fibrates, certain antioxidants such grape seed extract, quercetin and pterostilbene. These

studies confirmed the synergistic effects by enhancing antioxidant enzyme levels, scavenging free radicals in both animal models and human trials (21-24).

So far the pharmacokinetic profiles of selected compounds in animal and human models have been published in literature however, no data is reported in their concomitant use (25, 26). In current study a novel RP-HPLC based analytical technique was developed and successfully employed for the observing pharmacokinetic interactions between NA and PT following various treatment protocols.

Simultaneous investigation of hydrophilic and lipophilic compounds especially the naturally occurring compounds is difficult, and mostly reported individually with complex buffered mobile phases (27). Among various protocols gradient elution, mass spectrometry coupled with electron spray ionization, online and capillary hydrophilic interactions and specially fabricated stationary phase are well documented (28-31).

The quaternary ammonium compounds are well reported for retaining acidic solutes due to its amphiphilic nature (hydrophilic and lipophilic ends) (32, 33). However, is being never reported so far for simultaneous pharmacokinetic profiling of both natured compounds on reverse phase HPLC/UV with C18 column in clinical trials. There are already developed methods that utilized C18 stationary phase. Yet, combining with ion exchange method may provide better control of the retention time in case of hydrophilic moieties. In current method Hexa-decyltrimethylammonium bromide is utilized for ion-exchange for pharmacokinetic profiling of niacin and pterostilbene in biological samples.

2. MATERIALS AND METHODS

2.1. Chemicals

Pterostilbene (99.0% pure, Shanghai Korey Pharm Co., Ltd. China), Niacin (99.9% pure, Scharlau Chemie SA). Rosuvastatin used as internal standard (99.9% pure, Fluka-Chemika Switzerland). HPLC grade Acetonitrile (ACN) and methanol (MeOH), Hexa-decyltrimethylammonium bromide (cetrimide), formic acid were purchased from Sigma- Aldrich. Distilled water was prepared by Millipore distillation apparatus.

2.2. Instrumentation and Chromatographic conditions

Analysis was performed on Perkin Elmer HPLC system (Norwalk, USA) with UV/Vis detector (series 200), samples were eluted by Athena C18-WP (100A, 4.6mm × 250mm, 5 µm) HPLC column. A wavelength 250nm λ-max was selected and analysis was performed at ambient temperature using mobile phase 5mM cetrimide: organic phase (80:20 ACN: MeOH) in 30:70 at pH 2.5, pumped at 1mL min^{-1} isocraticaly.

2.3. Standard and sample preparations

Stock solutions of niacin (NA), pterostilbene (PT) and internal standard (IS) were prepared in methanol at concentration of 1mg $10mL^{-1}$, protected from light and stored at -20ºC for maximum stability. For calibration, serial dilutions of QC samples were made in mobile phase in 0.01µg mL^{-1}-20µg mL^{-1} concentration range.

To an aliquot of 200µL plasma sample in a plastic centrifuge tube 10µL of IS and 800µL mobile phase was added, vortex-mixed followed by centrifugation at 4 ºC for 10 minutes. A volume of 50µL supernatants were used for calibration curves and pharmacokinetic profiling.

2.4. Chromatographic condition optimization

Various chromatographic parameters such as mobile phase composition, pH and flow rate, analysis temperature, wavelength and internal standard were evaluated.

2.5. Method validation

Standard guidelines for specificity, linearity, accuracy and precision (repeatability), sensitivity, robustness, sample stability and suitability of chromatographic system (34, 35).

2.5.1. Specificity and linearity

The specificity was determined by various batches of 1:1 binary solution mixture in mobile-phase and blank

plasma. Each blank sample was evaluated for interferences and compared with those obtained from standard solutions. Data was recorded in 0.01-20μg mL^{-1} concentration range by constructing calibration curves plotted as analyte response ratios.

2.5.2. Precision and accuracy

Injection repeatability, analysis repeatability, and inter-day, intraday repeat analysis were conducted at 0.25, 0.50 and 1.0μgmL^{-1} for precision and 0.5, 1.0 and 2.0μgmL^{-1} for accuracy using the following equation.

$$\% \text{ Recovery} = \frac{\text{Peak response of analyte in spiked sample}}{\text{Peak response of analyte in mobile phase}} \times 100 \quad \text{eq: 1}$$

2.5.3. Sensitivity and stability of samples

Method sensitivity was assessed in terms of the lower limits of detection (LLOD) and lower limits of quantification (LLOQ). The QC samples were subjected to short-term to long-term storage conditions at ambient (25ºC), refrigerator (4 ºC) and at freezing (-20ºC) for 7 days. Stabilities were determined at three QC concentrations (0.5, 1 and 2 μg mL^{-1}) in triplicate.

2.5.4. Robustness and system suitability tests

Deliberate changes (±2%) in chromatographic conditions were made to observe method robustness. System suitability tests were performed on separation factor (α), retention factor (*k*), tailing factor (*T*), peak separation coefficient (*Rs*) and number of theoretical plates (*N*) for chromatographic system validation.

2.6. Pharmacokinetics of analytes in healthy human subjects

Ten healthy volunteers were recruited and informed consent was obtained regarding the experiment. Experiment procedure was approved from the ethical board of Pharmacy department, Sarhad University of Science and Information Technology, Peshawar bearing reference: 03/EC-22/Pharm following the principles of the Declaration of Helsinki. All subjects were confirmed of abstinence from other medications including smoking and snuff. Clinical data regarding physical examination, lipid profiles, urine analysis, and blood pressure were also monitored.

Pharmacokinetic study was performed according to randomized, open label, crossover design with a wash out period of one week between the treatments. Medical histories of all the subjects were evaluated and their biomedical profiles were recorded. Tablet dosage forms were developed and optimized containing 250mg of each analyte separately. Various formulation parameters including hardness, disintegration and dissolution tests were evaluated and optimum formulations were selected (data not shown). The subjects were administered with niacin and pterostilbene 250mg in single dose individual therapies and after washout period combination therapies (250mg niacin+ 250mg pterostilbene) was performed. Each study was conducted for seven days, and blood samples were collected in heparinized tubes at 1, 3, 5 and 7^{th} day. At day first and last the collection was performed at 15min, 30min, and at 1, 4, 8, 16 and 24hour. The plasma was separated at 4000×g at 4°C centrifugation and refrigerated till analysis.

2.7. Statistical tools

The pharmacokinetic parameters (max. plasma conc. (C_{max}), max. time (T_{max}), mean residence time (MRT) and plasma half-life ($t_{1/2}$)) were determined from the data. Students-*t* test was followed at 95% confidence interval to determine statistically significant differences between the therapies. The area under curve from zero to t (AUC_{0-t}) and infinity ($AUC_{0-\infty}$) for both drugs among individual and combine treatment groups were determined by linear trapezoidal method.

3. RESULTS

3.1. Extraction of analytes

Among solvents the mobile phase was able to recover analytes at optimum concentrations at composition of aqueous (5mM CTAB) and organic (80:20 ACN: MeOH) in ratio of 30:70 v/v as provided below (tab-1).

Table 1: Effect of extraction solvent on percent recoveries

Extraction solvent	Niacin *Mean percent ± SD*	Pterostilbene *Mean percent± SD*
MP	90.56 ± 1.56	96.38 ± 1.08
MeOH	85.42 ± 1.13	90.22 ± 1.19
ACN	75.27 ± 1.36	88.50 ± 1.04

3.2. Solvent composition

Results showed parallel increase in niacin retention time (RT) with CTAB concentration reaching maximum with 5mM L^{-1}, along with slight reduced pterostilbene RT. Optimizing organic phase, the acetonitrile and methanol individually produced no satisfactory results, however their combination (80:20, blue) produced sharp peaks and complete resolutions (Figure 3). In aqueous vs. organic ratios, the 30:70 (green) was found optimum for analysis at flow rate of 1mL min^{-1}.

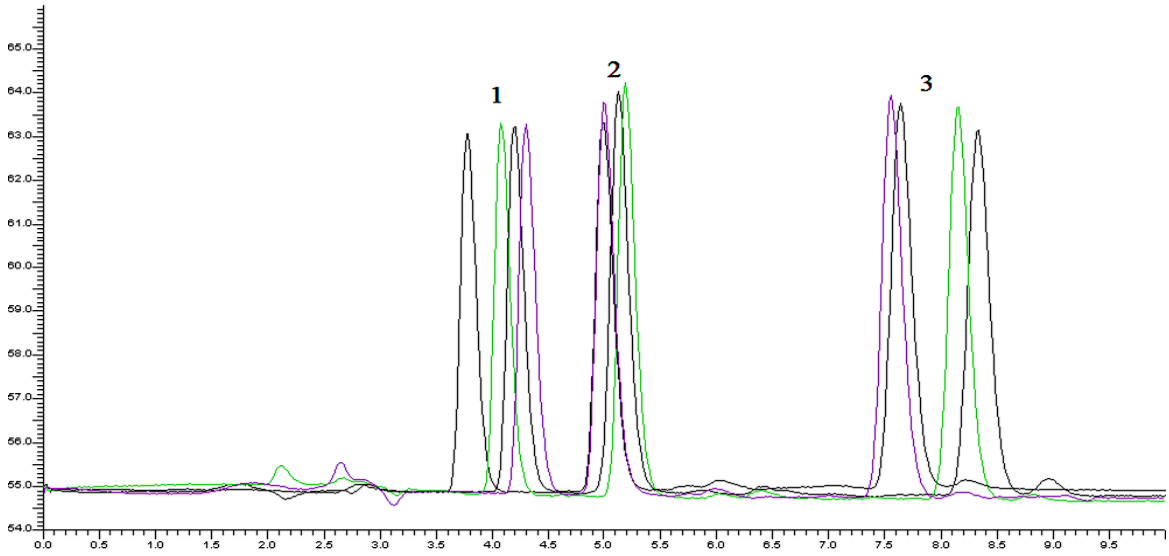

Figure 3: Optimizing organic phase ratio for analyte separation. (1) Niacin, (2) Internal Standard (3) Pterostilbene

Among various acids the formic acid (low MW) was selected for pH adjustment due to least effect on RT. The pH was adjusted to 2.5 (table 2).

Table 2: Retention time optimization

	Retention time in minutes		
	Niacin	**Pterostilbene**	**Rosuvastatin (IS)**
FA	4.28 ± 0.08	7.76 ± 0.04	5.14 ± 0.15
AA	2.68 ± 0.11	5.88 ± 0.07	2.91 ± 0.07
PA	1.98 ± 0.09	6.07 ± 0.13	5.78 ± 0.11
TCA	1.46 ± 0.16	5.39 ± 0.07	3.26 ± 0.09

3.3. Selection of column oven temperature and detector wavelength

Increased temperature ≥ 30ºC enhanced sensitivities but also resulted in poor resolution of IS and PT, with loss of NA retention. Therefore, the ambient temperature was selected for better results. The optimum peaks with zero interference were recorded at 250mn wavelength as shown in chromatograms of standard and spiked sample at 250nm in Figure 4.

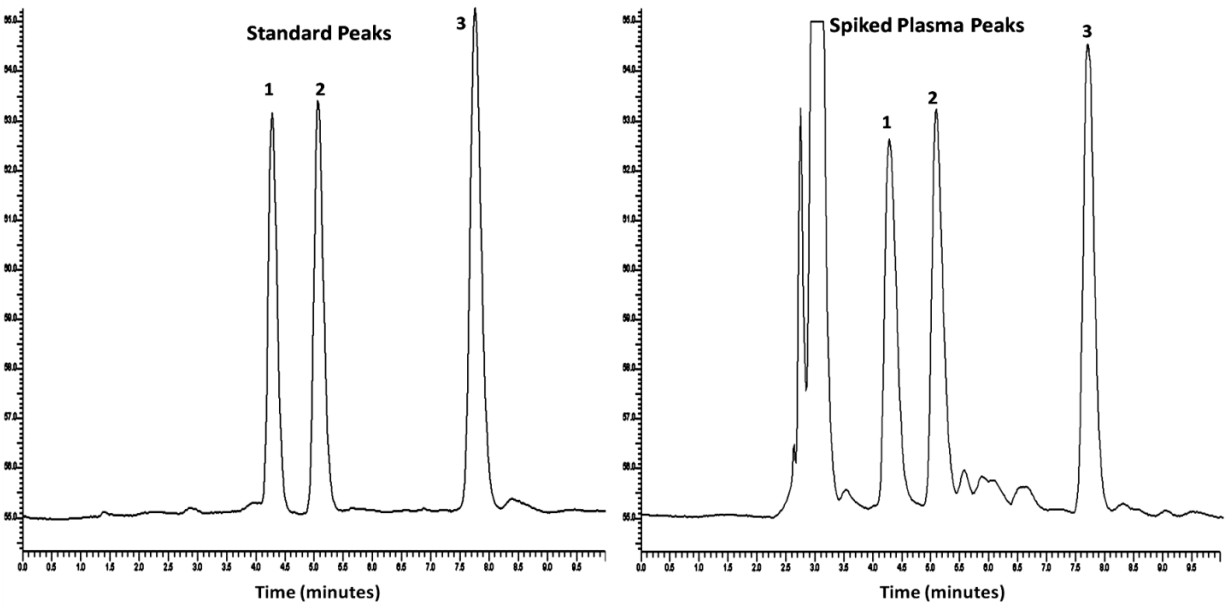

Figure 4: Chromatograms of (1) Niacin, (2) Internal Standard (3) Pterostilbene

3.4. Validation of proposed methods

3.4.1. Specificity and selectivity

As shown in the chromatograms of standard and spiked plasma in Figure 4, the retention time of NA, IS and PT were 4.28, 5.15 and 7.76min respectively. No significant interferences were observed from the blood endogenous substances.

3.4.2. Linearity

Acceptable linearity (r^2=0.999) was observed in standard and spiked samples. The slopes, intercepts and correlation coefficients (r) are summarized in table 3.

3.4.3. Precision and Accuracy

The precision tests were observed within acceptable ranges and developed the method was found precise and accurate (table 3).

3.4.4. Sensitivity

The quaternary ammonium amphiphilic ion-pairing agent was found appropriate for simultaneous quantification of opposite natured analytes sensitively in the intended biological matrices which is evident from LLOQ of NA and PT (50 and 22ng mL^{-1}).

3.4.5. Robustness

The developed method was robust, and negligibly effected by minor chromatographic changes. However, mobile phase composition was critical in efficient resolution of analytes.

Table 3: HPLC method linearity and accuracy evaluation

		Niacin	**Pterostilbene**
Linearity (μgmL^{-1})		0.020-20	0.020-20
Std. Regression		y= 0.8018x + 0.2868	y= 1.342x + 0.6412
Correlation (r)		0.9994	0.9998
Spiked Spl. Regression		y= 0.7277x + 0.1306	y= 1.267 + 0.2238
Correlation (r)		0.9999	0.9990
Accuracy (μgmL^{-1}) (% Recovery ± SD; % CV)			
0.5		90.42 ± 2.44; 2.61	96.80 ± 2.56; 2.64
1		91.65 ± 1.81; 1.91	97.31 ± 2.25; 2.31
2		90.57 ± 2.15; 2.29	95.85 ± 2.72; 2.84
Method Precision			
Injection repeatability ($1\mu gmL^{-1}$)			
Peak area ± SD; %CV		84265 ± 523; 0.62	135088 ± 1165; 0.86
RT ± SD (min); %CV		4.28 ± 0.08; 1.87	7.76 ± 0.04; 0.52
Analysis repeatability (μgmL^{-1})			
Spiked		0.5	0.5
Recovered ± SD; %CV		0.45 ± 0.058; 3.19	0.48 ± 0.064; 3.31
Repeatability studies (Spiked conc. in μgL^{-1})		**Recovered** (Mean ± SD; %CV)	
Intra-day	0.25	0.22 ± 0.01; 4.55	0.23 ± 0.01; 4.35
	0.50	0.43 ± 0.03; 6.98	0.46 ± 0.02; 4.35
	1.0	0.91 ± 0.02; 2.20	0.94 ± 0.03; 3.13
Intra-day	0.25	0.20 ± 0.01; 4.76	0.23 ± 0.01; 4.35
	0.50	0.43 ± 0.02; 4.65	0.44 ± 0.01; 2.27
	1.0	0.89 ± 0.04; 4.35	0.90 ± 0.03; 3.19
Detection limits ($ngmL^{-1}$)			
	LLOD	20	10
	LLOQ	50	22

3.4.6. Stability

Standard solutions of both analytes and IS were stable throughout the study duration at 4ºC and -20ºC. In case of spiked plasma samples, niacin stability decreased by 15% at 4ºC and 5% at -20ºC after two weeks.

3.4.7. Chromatographic System Suitability

The chromatographic system specification and test produced values were within boundaries and in accordance with the guidelines.

3.5. Pharmacokinetic observations

Mean plasma concentration vs time profile curves were established for niacin and pterostilbene. The extracted data is tabulated in table 4. In niacin therapies, we found insignificant differences in *Cmax, Tmax, $t_{1/2}$ and MRT* ($p>0.05$) among all single and multiple doses. The AUC in combined multidose was observed slightly higher but statistically insignificant ($p>0.05$). There was no significant variations observed among niacin AUCs in combined single vs. multidose therapies ($p>0.05$) and overall fluctuation in AUC_{0-24} observed was in 1545.75±142 to 1655±120 range. Similar findings were also reported in pharmacokinetic data on combined therapy of niacin and lovastatin, and a slight raised AUC in combined multidose was found insignificant (26). Pterostilbene pharmacokinetic data showed insignificant variations in *Cmax, Tmax, $t_{1/2}$, MRT and AUC* ($p>0.05$) among all treatment groups. Overall, the Cmax and t-half were fluctuated in range of 72.8±41 to 86.8±27 and 1.70 to 1.80 hours respectively (table 4).

These results suggested that both drugs in single and

multiple dose therapies have no effect on the pharmacokinetics of each other and hence no significant drug interaction on concomitant administration. Facial flushing with niacin was observed in some subjects but they recovered without treatment.

Table 4: Pharmacokinetic profiles of pterostilbene and niacin in single- and multi-dose administrations in individual and combination therapies (n=10)

Therapy	Type	C_{max} (ηgmL^{-1})	T_{max} (h)	$t_{1/2}$ (h)	MRT (h)	AUC_{0-t} (ηg.hr mL^{-1})	$AUC_{0-\infty}$ (ηg.hr mL^{-1})
NA (S)	**Monotherapy**	98.3 ±37	2.65 ±0.11	1.82 ±0.25	2.79 ±0.37	1545.75 ±142	1628.26 ±116
NA (M)		118.4 ±45	2.70 ±0.11	1.84 ±0.25	2.86 ±0.42	1620.65 ±122	1670.53 ±116
PT (S)		72.8 ±41	2.10 ±0.07	1.70 ±0.19	3.20 ±0.31	929.25 ±70	1004.41 ±83
PT (M)		85.5 ±28	2.15 ±0.04	1.71 ±0.17	3.62 ±0.36	994.25 ±84	1039.40 ±75
NA (S)	**Combine Therapy**	115.6 ±45	2.72 ±0.14	1.86 ±0.21	2.86 ±0.42	1615 ±128	1632.33 ±141
NA (M)		122.6 ±41	2.78 ±0.14	1.86 ±0.21	2.97 ±0.37	1655 ±120	1657.07 ±133
PT (S)		65 ±34	2.15 ±0.04	1.74 ±0.24	3.51 ±0.24	964.25 ±81	1039.40 ±75
PT (M)		86.8 ±27	2.18 ±0.04	1.80 ±0.18	3.62 ±0.36	1045.51 ±82	1077.53 ±102

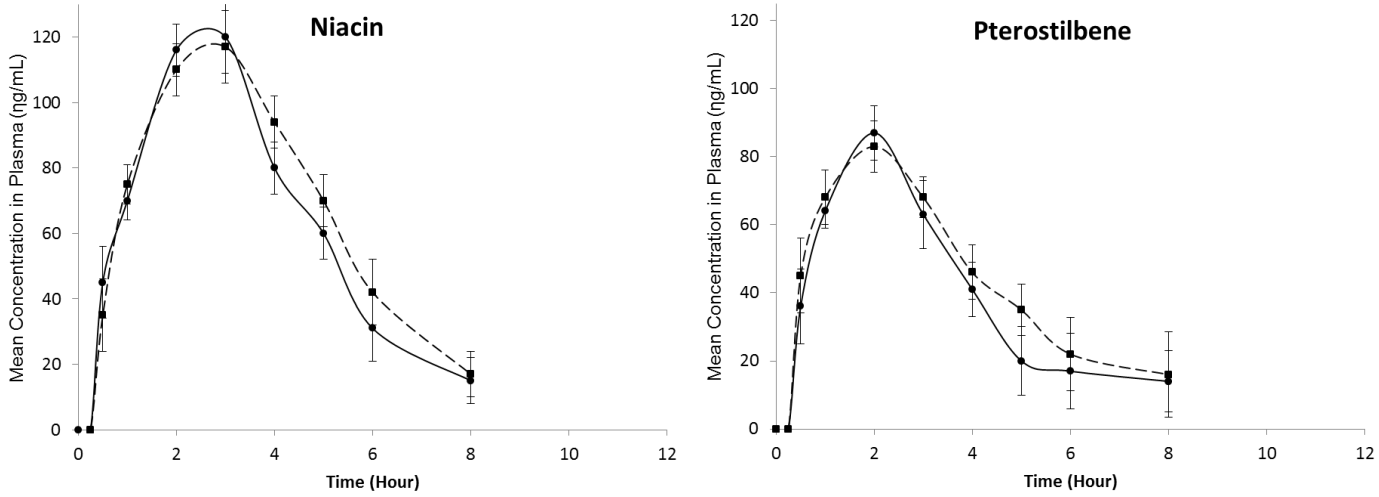

Figure 5: Mean plasma concentration vs. time curves for pterostilbene and niacin following oral administration. The continuous and dashed line shows multidose individual and combined drug administration respectively

4. DISCUSSION

Dietary antioxidants are very important for the processing of various functions in our daily life. They play major role in oxidative homeostasis. Various vitamins such as ascorbic acid, niacin, tocopherols and polyphenols (flavonoids, stilbenoids) are repeatedly used against oxidative stress. These agents' helps in scavenging reactive oxygen species (ROS) (hydroxyl, peroxy, alkoxyl-radicals, hydrogen peroxide) produced during cellular processes due to environmental and biological factors such as radiations, smoking, chemical exposure and excessive fatty diet. These species further produce detrimental effects such as, oxidation of low density lipoproteins, atherosclerosis, hypertension, neurodegenerative diseases. These natural antioxidants act dose dependently before reaching to the ceiling line, after which they can become prooxidant producing negative effects. The bioavailability studies of antioxidants are

mostly being reported individually, despite being used in combinations (36-39).

Some randomized clinical trials concluded no beneficial effects of antioxidant therapies in both individual and combinations and in one study increased total mortality rate was observed upon vitamin C, E and beta-carotene supplementation. Similarly, synergism was observed using pterostilbene with vitamin E in cancerous cells (40-42). All these results suggest that these effects of antioxidants combinations could be due to either prooxidant effect, unable to regenerate back to their reduced form or mechanisms other than antioxidizing. The synergism could be due to inhibiting their metabolic pathways thus increasing the plasma concentrations or uncommon mechanisms (43-45).

Besides evaluating the pharmacologic outcomes of combined treatments such as peroxidative end products, endogenous antioxidants levels i.e. glutathione, ascorbic acid and antioxidant enzymes, the pharmacokinetic interactions must also not be underestimated. Published data reported potent inhibitory effects of antioxidants on cytochrome P450 and subclasses. Myricetin, coumarins and some flavonoids were found to inhibit CYP3A4 and CYP2C9 in both *invivo* and *invitro* studies (46-48). Our method was found linear and accurate in quantifying the analytes with high sensitivity as tabulated in results. Comparing the AUC in all treatment groups insignificant variations were observed indicating no interaction between the antioxidants. To our knowledge no relevant data is reported regarding pharmacokinetics of antioxidants on concurrent use in human subjects.

From our study it can be concluded that concomitant use of both drugs in their recommended doses is therefore safe in indicated clinical conditions.

CONCLUSION

This study revealed that concurrent use of niacin and pterostilbene has no effect regarding inhibiting or enhancing drug metabolism that can either result in ceiling effect, initiate adverse effects or may lead to insufficient dose to get desired effects. Furthermore, clinical studies should be carried out before administering antioxidants in combined formulations or with other clinical drugs in general population to prevent emergence of any adverse reactions.

Author Contributions: Concept; P.A.H. and J.A.K.; methodology, S.A. and I.U.; software, F.S.; validation, R.U. and M.A.K.; analysis, P.A.H.; investigation, F.S.; writing, original draft preparation, P.A.H.; review and editing, R.U.; All authors have read and agreed to the current manuscript version.

Conflict of interest

The authors declare no conflict of interests.

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Ethical approval

The study was approved by the ethical committee Pharmacy department, Sarhad University of science and information technology, Peshawar (reference: 03/EC-22/Pharm approved on 24th May 2022).

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التقييم الدوائي الحركي للنياسين والبتيروستيلبين في الجرعات المفردة والمتعددة لدى الأشخاص الأصحاء

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ملخص

تمت دراسة إمكانات خلطات مضادات الأكسدة الطبيعية في متلازمات التمثيل الغذائي والأمراض الأخرى بشكل متكرر.

الخلفية: تهدف هذه الدراسة غالبية الدراسات على التفاعلات الدوائية، في حين أن الدراسات حول تفاعلاتها الدوائية الحركية محدودة تعتمد إلى تقديم رؤية حول التفاعلات الدوائية الحركية بين النياسين والبتيروستيلبين عند تناولهما معًا، بالإضافة إلى قياس تركيزاتهما في الدم الجرعات المفردة والمتعددة عند (PT) والبتيروستيلبين (NA) تم اتباع تصميم عشوائي، مفتوح التسمية، ومتقاطع لدراسة التفاعل الدوائي الحركي بين النياسين.

المنهجية :تم إعطاء المشاركين جرعات مفردة ومتعددة (250 ملغ لكل جرعة) . في تركيبات الجرعات المفردة والمتعددة لدى متطوعين أصحاء حديثًا للتقدير المطورة HPLC-UV طريقة تم جمع عينات الدم وتحليلها باستخدام. بشكل فردي وفي تركيبات لمدة أسبوع واحد مع استخدام هيكسا–ديسيل تري ميثيل أمونيوم بروميد كعامل اقتران أيوني الكمي المتزامن، حيث كانت حدود التقدير وجد أن طريقتنا خطية ضمن نطاق التركيز المطبق (0.020–20 ميكروغرام/مل) وحساسة

النتائج: أظهر التحليل الدوائي الحركي .على التوالي (PT) والبتيروستيلبين (NA) الدنيا 50 و22 نانوغرام/مل لكل من النياسين للمركبات على بعضها البعض في .لمضادات الأكسدة المُعطاة عدم وجود تأثير كبير (Cmax Tmax)، AUC، MRT، و (t1/2 عند مقارنة الجرعات الفردية مقابل التركيبات، لوحظت تباينات غير دالة إحصائيًا. كل من العلاجات ذات الجرعة المفردة والمتعددة .في تركيزات الأدوية في البلازما ($p>0.05$) أظهرت نتائج هذه الدراسة التوافق الحيوي للأدوية المختبرة كما تم إثباته من خلال البيانات الدوائية الحركية، وبالتالي يمكن استخدامها بأمان ضمن الجرعات الموصى بها في التركيبات المشتركة.

الكلمات الدالة: الأحماض الكربوكسيلية؛ مضادات الأكسدة الطبيعية؛ المساحة تحت المنحنى ؛RP-HPLC-UV.

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In Silico Antioxidant Activity of Six Volatile Constituents in *Capsella bursa-pastoris*

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ABSTRACT

Capsella bursa-pastoris is a wild herb with high nutritional value that can be eaten raw or cooked in some countries. It is also used in the traditional medicine of many countries as an anti-bleeding agent and to relieve inflammation. This study aimed to identify the chemical composition of essential oil and assess the in silico antioxidant activity of six volatile constituents in *Capsella bursa-pastoris* grown in Syria. The essential oil was extracted and analyzed using gas chromatography-mass spectrometry (GC-MS). In addition, in silico pharmacokinetics and molecular docking of six volatile constituents (Phytone, Phytol, Farnesylacetone, Octa-3,5-dien-2-one, m-menthane, and beta-ionone) were performed on Xanthine oxidase (PDB ID: 1 FIQ). The results revealed the presence of thirty-eight compounds. The main compounds were hexahydrofarnesyl acetone (Phytone) at 20.2%, diacetyl-4,4',6,6'-tetramethoxy-2,2'-biphenyldiol at 8.46%, diisopropyl methylphosphonate at 6.45%, and beta-ionone at 5.24%. Farnesyl acetone and beta-ionone exhibited the highest binding affinity, ranging from -5.4 to -6.4 kcal/mol. The essential oil of *Capsella bursa-pastoris* is a potential source of antioxidants.

Keywords: *Capsella bursa-pastoris*, essential oil, antioxidant, molecular docking.

INTRODUCTION

Capsella bursa-pastoris, is one of the most important and widespread species in the genus Capsella. It belongs to the Brassicaceae family. *C. bursa-pastoris* is a small annual wild herb with a global distribution. It is distinguished by its inverted, triangular fruits that resemble a shepherd's purse. Hence its common name: Shepherd's purse[(1)].

C. bursa-pastoris grows in all parts of the world except tropical regions. The aerial parts are the medicinal component of the plant. The height of the plant is 2-40 cm. It has a simple, erect stem and a simple spindle root. The small flowers are distinguished by their white color and have four sepals, four petals, and six stamens. Its fruits are in the form of a green capsule containing many reddish-brown seeds[(2)].

C. bursa-pastoris has high nutritional value. In some countries, it is eaten fresh or cooked, commonly used as a salad ingredient[(3)]. It has been a staple in the diets of people in China and Japan for centuries[(4)] The plant is used internally for mild menstrual disorders such as menopause, and externally for nasal bleeds and bleeding surface skin wounds[(2)].

Studies have shown the presence of flavonoids and alkaloids, as well as minerals, vitamins A, C, and B (1, 2, 3, 6), and unsaturated fatty acids. It also contains many amino acids and organic acids (including formic, quinic, and caffeic), as well as glucosinolate, the production of which is characteristic of *C. bursa-pastoris*[(4)].

Studies have also shown that *C. bursa-pastoris* has anti-inflammatory[(5,6)] and antibacterial[(7)] efficacy, as well as antioxidant[(8)] and tumor growth inhibitory[(9)] effects.

This study aimed to identify the chemical composition

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of essential oil and assess the in silico antioxidant potential of major volatile constituents in *C. bursa-pastoris*.

MATERIALS AND METHODS:

Plant Material

The aerial parts of *C. bursa-pastoris* were collected in April 2020 from the park of the Faculty of Science at Damascus. The plant was identified and classified. The samples were dried immediately after collection by air drying at normal room temperature, away from sunlight.

Extraction

The essential oil was extracted from 90 grams of plant material using the steam distillation method.

Analysis of Essential Oil Ingredients

The analysis of the essential oil components was conducted according previous study[3], using Gas Chromatography-Mass Spectrometry (GC/MS) (Agilent Tecnologies 5957C). The following thermal program was applied: 60°C for 1 minute, increased to 70°C at 4°C/min, then to 130°C at 5°C/min, followed by 160°C at 4°C/min, then to 220°C at 5°C/min, and finally to 280°C at 15°C/min, with a total operation time of 44 minutes. Helium gas was used as the mobile phase at a flow rate of 1 mL/min and a pressure of 8.2317 Pa. The column used was HP-5MS 5% Phenyl Methyl Silox, with dimensions of 30 m x 250 µm x 0.25 µm, operated at 325 °C. Spectra were determined based on the NIST library (National Institute of Standards and Technology).

Drug Likeness and ADMET Properties

Drug-likeness rules are a set of guidelines for the fundamental properties of compounds. They are used for the rapid calculation of drug-like properties of a chemical compound. These rules are not supreme, nor do they aim to establish strict cutoff values to determine which property values are considered drug-like and which are not. However, they can be very viable and productive[10]. In this study, we utilized Swiss ADME to calculate drug likeness and pharmacokinetic properties[11], while toxicity risks were predicted using Osiris software[12].

Swiss ADME is an open-source virtual screening tool. Its calculation is based on various drug-likeness criteria such as Lipinski's rule[13], Ghose filter[14], Veber rule[15], Egan[16], and Muegge rule[17].

Swiss ADME was also used for predicting biological activity. It is a simple, accurate, and robust tool that enables the understanding of ADME properties of a compound. The parameters it provides include gastrointestinal absorption (GI) and permeation through the blood-brain barrier (BBB)[18]. The efflux pump P-glycoprotein, involved in pharmacokinetics and drug interactions, is an important parameter in discovery screening, particularly for drugs that need to penetrate the brain[19]. It is embedded in the Swiss ADME suite and built upon by the Support Vector Machine (SVM) model. The Swiss ADME package calculates the ability of a specific molecule to inhibit key cytochrome enzymes involved in drug metabolism[20].

Structures of the tested compounds were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and SMILES notations were copied and used as query files in the Swiss ADME tool.

The determination of the toxicity of chemical compounds is crucial for understanding their harmful effects on humans, animals, plants, and the environment. In vivo animal testing is limited by time, ethical considerations, and financial obstacles[21]. Computational methods are considered useful for assessing the toxicity of chemicals. In silico toxicology is proposed to assist in ongoing toxicity testing to predict toxicity, prioritize chemicals, guide toxicity testing, and minimize failures in the later stages of the drug design process[22]. The web tool Osiris Property Explorer was used to explore toxicity risks (https://www.organic-chemistry.org/prog/peo/). It is an easy and free tool to infer physicochemical and toxicological molecular properties. It provides the ability to estimate the risk of adverse effects, such as mutagenic, tumorigenic, irritant, and reproductive effects, as well as relevant drug properties, and an overall drug likeness

score. Mutagenicity structural alert refers to a set of molecular functions or substructures linked to the mutagenic activity of chemical compounds[23]. Several functional groups in chemical structures are suspected to be associated with tumorigenic effects[24]. The Osiris software enables the prediction of tumorigenic potentials, as well as irritant and reproductive effects, and provides a quantitative estimate of drug-likeness (QED).

Molecular Docking

Molecular docking is a technique that predicts the optimal binding mode of a ligand to a macromolecule[25]. The utilization of molecular docking in the process of natural products-based drug discovery provides insights into elucidating traditional uses and potentially identifying new applications for existing medicinal plants[26].

A molecular docking study was conducted using AutoDock Vina. It is one of the most popular protein-ligand docking software, besides being freely available and an open-source tool[27]. It provides a significant improvement in accuracy and a two-order magnitude of speed compared to AutoDock4. Molecular interactions were investigated for the compounds Phytone, Phytol, Farnesylacetone, Octa-3,5-dien-2-one, m-Menthane, Beta-ionone, and ascorbic acid as a reference compound. The process of molecular docking of our tested compounds went through the following steps:

Protein Preparation

The three-dimensional (3D) X-ray crystallographic structure of Xanthine oxidase was downloaded from the PDB website (https://www.rcsb.org/structure/1FIQ) with the ID: 1FIQ solved at a resolution of 2.5 Å.

The Protein Data Bank (PDB) file. The crystal structure of the enzyme is complexed with small molecules; therefore, the active site was emptied of all heteromolecules as a first step. Water molecules were removed, and all hydrogen atoms were added. Eliminating water molecules is essential because they can form unnecessary bonds with the ligand[28]. Kollman charges were added and spread on the protein, and there were no missing atoms to be added. The protein file was saved as a PDB file. Autodock Vina deals with PDBQT files. Consequently, the protein file was converted to PDBQT file format and saved for the docking process.

Ligand Preparation

The files of the studied compounds were downloaded from the https://pubchem.ncbi.nlm.nih.gov/ database as SDF files. The files were converted to PDB format using the OpenBabel software. To convert to PDBQT files, the torsion tree was selected, and rotation flexibility was maintained by default. Gasteiger charges were also added, and nonpolar hydrogens were merged. The structures were finally prepared for the docking process.

Grid Box Defining

The grid box position defines the space within the protein where the docking will take place. The grid box has dimensions of 40 x 40 x 40 units with a grid spacing of 0.375 units[29].

Docking Analysis

Once the docking was completed, the ligand poses with equal or lower affinity than the reference inhibitor, and root mean square deviation (RMSD) values lower than 2.5 Å were considered as potential inhibitors. To assist in the calculations and analysis of the results, the Protein-Ligand Interaction Profiler (PLIP) was used[30].

RESULTS AND DISCUSSION

The study focused on investigating the chemical composition of essential oil and the in silico antioxidant activity of major volatile constituents in *Capsella bursa-pastoris* .

Gas chromatography-mass spectrometry (GC-MS) analysis in Fig. 1 of the essential oil revealed the presence of thirty-eight compounds as shown in Table 1. The essential oil of C. bursa-pastoris contains various components: hydrogen carbonates constitute 19.25%, ketone compounds 23.9%, aldehydes 2.66%, terpenoid and terpenoid compounds 4.65%, and esters 28.22%. Among the most important ketone compounds is

Hexahydrofarnesyl acetone (Phytone), which constitutes the highest percentage in the oil at 20.2%. It is a ketone compound that may potentially demonstrate antibacterial activity against both gram-positive bacteria against both gram-positive and gram-negative bacteria[31]. Additionally, 3,5-Octadien-2-one is utilized as a flavoring agent and food additive[32]. Among the terpene compounds, Beta-ionone (5.24%) is a monoterpene with a structure similar to the cyclic structure of beta-carotene. Some studies have shown that it has anti-tumor activity[33] and is used in the manufacture of vitamins A, E, and K1[34].

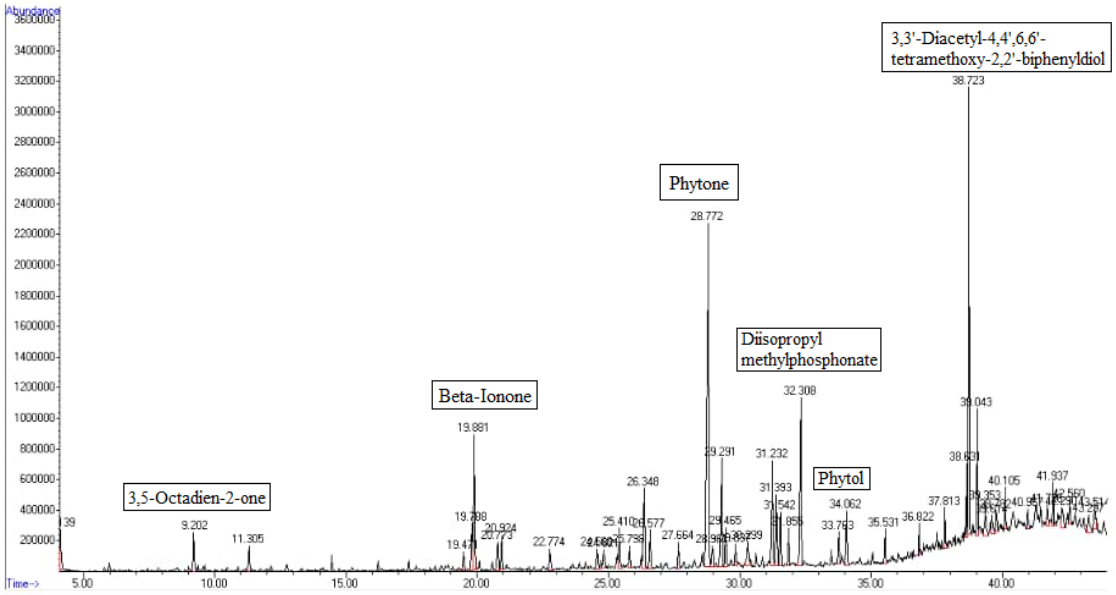


Fig. 1. GC-MS chromatogram of the essential oil of *Capsella bursa-pastoris*

Phytol (1.92%), an oxygenated diterpene, exhibits antibacterial activity and is utilized in the production of certain cosmetics, soaps, and detergents[31]. Farnesyl acetone (1.17%), a ketone terpenoid, and Menthane (1.55%), a monoterpenoid, are both utilized as flavoring agents and food additives[35]. When comparing the results of the analysis of essential oils in this study with other studies, it appears that there are some differences in the components of the oils, in addition to the presence of common components. **Table 2** displays the names of some common components along with a comparison of their percentage in the oil across different studies.

Drug Likeness and ADMET Properties

Drug Likeness and Bioavailability

The results obtained from the Swiss ADME suite showed that all the tested compounds followed the Lipinski rule, except for Beta ionone. However, only Beta ionone met the Ghose filter criteria. The concern was related to the logarithm of the partition coefficient (MLOGP) calculated using a topological method. The Ghose filter is a knowledge-based tool that aims to offer a qualitative and quantitative representation of drug-like chemical space for users. It can be utilized in drug discovery by assisting in the design of medicinal chemistry

or combinatorial libraries. The rules defined by this filter were as follows: 160 < molecular weight (MW) < 480, −0.4 < logP < 5.6, 20 < number of atoms < 70, and 40 < molar refractivity (MR) < 130[41].

Table 1. components of the essential oil compounds of *Capsella bursa-pastoris*

Peak no.	Compound	Peak area %	Retention time
1	3,5-Octadien-2-one	1.182	9.20
2	Methylcyclodecane	0.86	19.47
3	[(S)-2-nitro-2-cyclohexenyl]-acetate	1.75	19.79
4	Beta-Ionone	5.25	19.88
5	Cyclohexanecarboxylic acid, 4-cyanophenyl ester	1.27	20.77
6	2-Hydroxy-5-methoxybenzyl vinyl ether	1.21	20.92
7	Hexadecane	0.92	22.78
8	1-Heptadecene	0.92	24.82
9	Menthane	1.55	25.41
10	trans-Bicyclo[4.3.1]decan-10-one	3.68	26.35
11	Cinnamaldehyde, .alpha.-hexyl-	1.46	26.58
12	Octadecane	0.95	27.66
13	Phytone	20.21	28.77
14	Diisobutyl phthalate$$ 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	4.17	29.29
15	Cyclotetradecane	1.40	29.46
16	Nonadecane	0.77	29.84
17	Farnesyl acetone c	1.17	30.30
18	Dibutyl phthalate	4.16	31.23
19	Cyclohexane, 1-(cyclohexylmethyl)-2-methyl-, cis-	2.54	31.39
20	Sulfurous acid, cyclohexylmethyl hexyl ester	1.89	31.54
21	Eicosane	1.32	31.85
22	Diisopropyl methylphosphonate	6.45	32.31
23	Heneicosane	1.05	33.76
24	Phytol	1.93	34.06
25	Docosane	1.02	35.53
26	Tetracosane	0.84	37.81
27	Pentacosane	1.45	38.63
28	-3,3'-Diacetyl-4,4',6,6'-tetramethoxy-2,2'-biphenyldiol	8.46	38.72
29	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	3.08	39.04
30	9-Octylheptadecane	0.87	39.61
31	Heptacosane	0.95	39.78
32	Octacosane	0.77	40.97
33	Cholestane	0.91	41.72
34	Nonacosane	1.34	41.93
35	Pentanamide, N-(2-methyl-3-trifluoromethyl)phenyl-	1.25	42.29
36	i-Propyl 5,9,17-hexacosatrienoate	0.82	42.56
37	Stigmastane	1.11	43.30
38	5-[5'-Ethynyl(thien-2'-yl)]thiophene-2-carbaldehyde	1.21	43.51

Table 2. Comparison of essential oil components between the current study and other studies

Components	Area Pct						
	current study	Gao *et al*[36]	Choi *et al.* [37]	LIU Yu *et al.*[38]	Miyazawa *et al.*[39]	Lee *et al.*[40]	Gümüşok *et al.*[3]
3,5-Octadien-2-one	1.18		0.78			0.54	
Beta-Ionone	5.24		0.18			1.24	0.2
Hexadecane $$ n-Cetane	0.92				1.5		
1-Heptadecene $$ Hexahydroaplotaxene	0.92				0.3		
Octadecane	0.95		0.1		0.3		
Hexahydrofarnesyl acetone $$ Phytone	20.2	9.6	1.21	10.15		6.11	
Nonadecane	0.76		0.2				19.6
Eicosane	1.31				0.3	1.14	
Phytol	1.92	18	21.12			7.57	19.3
Tetracosane	0.84					4.84	
Pentacosane	1.44		0.65				13.5
Heptacosane	0.94		0.96				9.9
Octacosane	0.76			4.73			

Neither Phytone nor Phytol adhere to Veber and Egan filters. The Veber filter states only two rules regarding polar surface area (PSA) and the number of rotatable bonds (Rotors), which should not exceed 140 Å2 and 10, respectively[42]. The Egan filter utilizes multivariate statistics to assess membrane permeability for poorly absorbed compounds. LOGP and TPSA are the chosen descriptors in the Egan filter[43]. Both Phytone and Phytol exhibited a calculated WLOGP (calculated by atomistic method) of more than 5.8. The compounds were non-drug-like according to the Muegge rule. The nominated drugs must acquire two to seven pharmacophore points to pass the Muegge filter[44]. Violations occurred when the molecular weight (MW) was less than 200 and when XLOGP values were calculated using both atomistic and knowledge-based methods. All the investigated compounds exhibited moderate bioavailability, with values exceeding 50%. Results of drug likeness are presented in **Table 3**.

Pharmacokinetics and ADME Properties

The prediction of gastrointestinal absorption (GI) using in silico models has emerged as a widely used and promising complement to traditional in vitro assays[45]. With the exception of Phytol and m-Menthane, all the tested compounds demonstrated good gastrointestinal absorption. The blood-brain barrier permeability filters determine which compounds are included or excluded in both central nervous system (CNS) and non-CNS drug development processes[18]. Phytone, Phytol, and Farnesylacetone showed no ability to penetrate the blood-brain barrier (BBB), while Octa-3,5-dien-2-one, m-Menthane, and Beta-ionone are expected to cross the BBB. P-glycoprotein is a crucial factor in discovery screening, especially for drugs that need to penetrate the blood-brain barrier[46]. Only Phytone and Phytol are considered substrates for P-gp. Phytone, Phytol, Farnesylacetone, and m-Menthane are expected to inhibit CYP2C9. Farnesylacetone was found in a previous in silico study to exhibit convenient pharmacokinetics and bioavailability due to the polar carbonyl moiety[47]. Among the bioactive compounds studied, Octa-3,5-dien-2-one and Beta-ionone did not inhibit any CYP isoform, while Phytone, Phytol, Farnesylacetone, and m-Menthane were moderately metabolized as they only inhibited specific liver isozymes.

Previous computational analysis of the pharmacokinetic profile of phytol revealed that it does not permeate the blood-brain barrier (BBB) or the skin[48]. **Table 4** presents the pharmacokinetic properties of the bioactive components of Phytone, Phytol, Farnesylacetone, Octa-3,5-dien-2-one, m-menthane, and Beta-ionone.

Table 3: Drug Likeliness of the Selected Phytoconstituents from *Capsella bursa-pastoris* ….

	Lipinski	Ghose	Veber	Egan	Muegge	Bioavailability Score
Phytone	Yes; 1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	No; 1 violation: Rotors>10	No; 1 violation: WLOGP>5.88	No; 2 violations: XLOGP3>5, Heteroatoms<2	0.55
Phytol	Yes; 1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	No; 1 violation: Rotors>10	No; 1 violation: WLOGP>5.88	No; 2 violations: XLOGP3>5, Heteroatoms<2	0.55
Farnesylacetone	Yes; 1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	Yes	Yes	No	0.55
Octa-3,5-dien-2-one	Yes; 0 violation	No; 2 violations: MW<160, MR<40	Yes	Yes	No; 2 violations: MW<200, Heteroatoms<2	0.55
m-Menthane	Yes; 1 violation: MLOGP>4.15	No; 1 violation: MW<160	Yes	Yes	No; 2 violations: MW<200, Heteroatoms<2	0.55
Beta ionone	Yes	Yes	Yes	Yes	No: 2 violations: MW<200, Heteroatoms<2	0.55
Ascorbic acid	Yes; 0 violation	No; 2 violations: WLOGP<-0.4, MR<40	Yes	Yes	No; 1 violation: MW<200	0.56

Table 4. Pharmacokinetic properties of the bioactive components of Phytone, Phytol, Farnesylacetone, Octa-3,5-dien-2-one, m-menthane, and Beta ionone

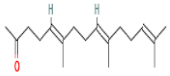
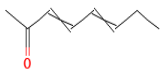
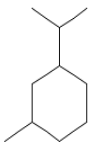
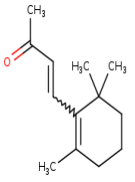
	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 Inhibitor	CYP3A4 inhibitor	Log Kp (skin permeation)
Phytone	High	No	Yes	No	No	Yes	No	No	-3.00
Phytol	Low	No	Yes	No	No	Yes	No	No	-2.29
Farnesylacetone	High	No	No	Yes	No	Yes	No	No	-3.95
Octa-3,5-dien-2-one	High	Yes	No	No	No	No	No	No	-5.74
m-Menthane	Low	Yes	No	No	No	Yes	No	No	-3.93
Beta ionone	High	Yes	No	No	No	No	No	No	-5.41
Ascorbic acid	High	No	No	No	No	No	No	No	-8.54

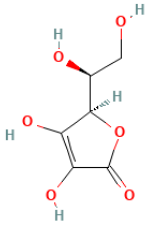
Toxicity risks

The key substructures responsible for certain toxicity are known as structural alerts (SAs). Medicinal chemists can rely on them during structural optimization to reduce the risk, as they are directly linked to toxicity. Certain substructures are associated with mutagenicity, while others are linked to carcinogenicity[49]. Octa-3,5-dien-2-one has mutagenic potential characteristics in addition to tumorigenic ones. Risk assessment of eye and skin irritation is crucial in the cosmetics and pharmaceutical

industries. Except for Phytol and m-menthane, the other tested constituents have a moderate risk of potential irritant properties. **Table 4** Essential ingredients chemical structures with their physicochemical properties. The toxicity risk results are represented in **Table 5**.

Table 5. The toxicity risks of Phytone, Phytol, Farnesylacetone, Octa-3,5-dien-2-one, m-menthane, Beta-ionone.

	Mutagenic	Tumorigenic	Irritant	Reproductive effects	Structure	Drug likeness	MW
Phytone CC(C)CCCC(C)CCCC(C)CCCC(=O)C	No	No	Yes	No		-7.06	268.48
Phytol CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C	No	No	No	No		-3.77	296.53
Farnesylacetone CC(=CCCC(=CCCC(=CCCC(=O)C)C)C)C	No	No	Yes	No		-4.59	262.43
Octa-3,5-dien-2-one CCC=CC=CC(=O)C	Medium Risk	Yes	Yes	No		-5.92	124.18
m-menthane CC1CCCC(C1)C(C)C	No	No	No	No		-6.17	140.27
Beta ionone CC1=C(C(CCC1)(C)C)C=CC(=O)C	No	High Risk	Yes	No		-6.41	192.30

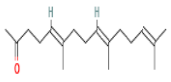
	Mutagenic	Tumorigenic	Irritant	Reproductive effects	Structure	Drug likeness	MW
Ascorbic acid C(C(C1C(=C(C(=O)O1)O)O)O)O							176.12

Molecular Docking

Molecular docking plays a vital role in drug discovery, where natural components from herbal origins are the main source of drugs[50].

The molecular docking of Phytone, Phytol, Farnesylacetone, Octa-3,5-dien-2-one, m-menthane, and Beta-ionone was performed on Xanthine oxidase (PDB ID: 1FIQ). We analyzed the active phytochemical compounds using binding free energy scores and molecular interaction profiles. The results of molecular docking and amino acid interactions are represented in **Table 7**. Farnesyl acetone and Beta-ionone displayed the best binding affinity, ranging from -5.4 to -6.4 kcal/mol. Ascorbic acid was utilized as a reference antioxidant, and the binding affinity of the bioactive components was compared to it. Phytone, farnesyl acetone, and beta-ionone showed higher affinity compared to ascorbic acid. Python, in turn, formed hydrogen bonds with some residues in the active site of the enzyme. Although lower than the affinity of ascorbic acid, phytol, octa-3,5-dien-2-one, and m-menthane showed binding affinities very close to that of ascorbic acid.

Table 6. The toxicitTable 6. Essential ingredients chemical structures with their physicochemical properties

Name	Structure	MW	BP	Density	Refraxtive index
Phytone		268.48	-	-	-
Phytol		296.53	202.00 to 204.00 °C	-	-
Farnesylacetone		262.43	-	0.885-0.895	1.478-1.483

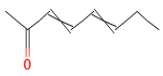
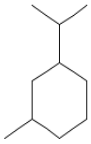
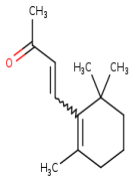
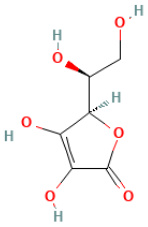
Name	Structure	MW	BP	Density	Refraxtive index
Octa-3,5-dien-2-one		124.18	-	0.880-0.890	1.508-1.516
m-menthane		140.27	-	-	-
Beta ionone		192.30	271 °C	-	-
Ascorbic acid		176.12	-	0.9461	1.517-1.522

Table 7. Molecular Docking results: Binding affinity and amino acid interactions

Ligand	Binding Affinity, ΔG (Kcal/mol)	Hydrogen-Bond Interactions	Hydrophobic Interactions	Salt Bridges
Phytone	-6.3	VAL259, ASN261	LEU257, 287, 404, GLU263, ILE264, 353	
Phytol	-5.6	ARG32, ASP594	LEU27,41, PHE604, ALA678, MET826	
Farnesylacetone	-6.4		LEU257, 398, ILE264, 353, ALA302, GLU263	
Octa-3,5-dien-2-one	-5.4		LEU257, 245, 287, ILE284, ALA301, 302, VAL259	
m-menthane	-5.6		ILE353, 398, ILE353	
Beta ionone	-6.4		LEU257, 398, ILE353	
Ascorbic acid	-5.9	LEU605, GLU676, 679, ALA678, MET826		ARG32

Previous studies proved interactions to be through hydrogen bonding and hydrophobic forces[51]. Phenylalanine, glutamate and arginine are key residues at the active site of the enzyme[52]. Fig. 2 represents the 3D binding mode of the phytoconstituents with the active site of 1 FIQ. Many Natural products showed antioxidant activity[53-55].

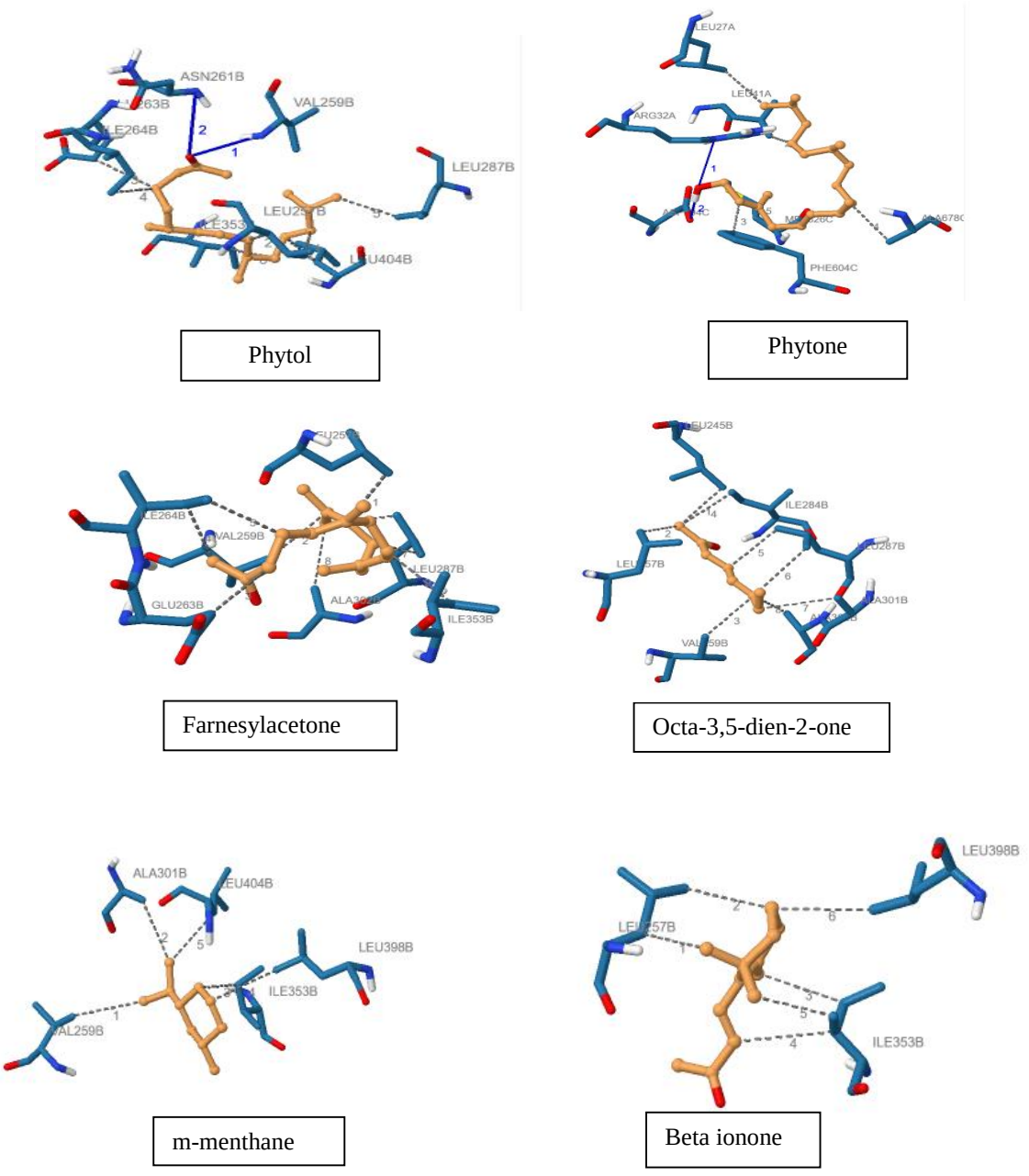


Fig 2. Binding mode of the phytochemical components with the active site of 1 FIQ

CONCLUSIONS

The in silico study demonstrated the antioxidant activity of farnesyl acetone and beta-ionone, which exhibited binding affinity to Xanthine oxidase. This suggests that they possessed important biological activities that require evaluation in the future.

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النشاط المضاد للأكسدة في الحاسوب لستة مكونات متطايرة في نبات *Capsella bursa-pastoris*

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ملخص

يعتبر نبات *Capsella bursa-pastoris* عشبة برية ذات قيمة غذائية عالية ويمكن تناولها نيئة أو مطبوخة في بعض البلدان. كما يستخدم في الطب التقليدي في العديد من البلدان كعامل مضاد للنزيف وتخفيف الالتهاب. هدفت هذه الدراسة إلى تحديد التركيب الكيميائي للزيت العطري وتقييم النشاط المضاد للأكسدة في الحاسوب لستة مكونات متطايرة في نبات *Capsella bursa-pastoris* المزروع في سوريا. تم استخلاص الزيت العطري وتحليله باستخدام كروماتوغرافيا الغاز–مطياف الكتلة (GC-MS). بالإضافة إلى ذلك، تم إجراء الحركية الدوائية الحاسوبية والالتحام الجزيئي لستة مكونات متطايرة (Phytone و Phytol و Farnesylacetone و Octa-3,5-dien-2-one و m-menthane و Beta ionone) على أكسيداز الزانثين (PDB ID: 1 FIQ). أظهرت النتائج وجود ثمانية وثلاثين مركبًا. كانت المركبات الرئيسية هي أسيتون هيكساهيدروفارنيسيل (فيتون) بنسبة 20.2٪، وداي أسيتيل–4،4'،6،6'–تيتراميثوكسي–2،2'–بيفينيل ديول بنسبة 8.46٪، وثنائي إيزوبروبيل ميثيل فوسفونات بنسبة 6.45٪، وبيتا أيونون بنسبة 5.24٪. أظهر أسيتون فارنيسيل وبيتا أيونون أعلى تقارب ارتباط، يتراوح من –5.4 إلى –6.4 كيلو كالوري / مول. يعد الزيت العطري لـ *Capsella bursa-pastoris* مصدرًا محتملًا لمضادات الأكسدة.

الكلمات الدالة: *Capsella bursa-pastoris*، الزيت العطري، مضاد الأكسدة، الالتحام الجزيئي.

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Polypharmacy in Type 2 Diabetes Patients of the PROLANIS Program in Indonesia: Identification of Potential Drug-Drug Interaction

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ABSTRACT

The identification of potential drug-drug interactions (pDDIs) becomes critical in evaluating medication safety among diabetes mellitus patients. This study aimed to identify the pDDIs of polypharmacy in type 2 diabetes mellitus (T2D) patients of the Chronic Disease Management Program or Program Pengelolaan Penyakit Kronis (PROLANIS) Program. The T2D patients aged ≥18 were selected consecutively. A total sample of prescriptions containing ≥5 drugs was included. The mean age of patients was 62.70 ± 9.85 years (range 24–92 years), 62% were elderly, and 56.8% were females. Polypharmacy prescriptions were most prevalent in the Internal Medicine Department (92.8%). Of the 250 prescriptions, approximately 78.4% contained at least one pDDI. A total of 515 pDDIs were identified, with a median of 2 pDDIs per patient. Of these, 89.7% were of moderate severity. The drug pairs involved in moderate-severity pDDIs were glimepiride-metformin, glimepiride-bisoprolol, and metformin-ramipril. The number of drugs per prescription is a significant predictor of pDDIs (aOR = 7.48; 95% CI = 1.73-32.32). Subsequent analysis revealed that prescriptions containing eight or more drugs were 4.31 times more likely to have more than five pDDIs (p=0.010). Pharmacists must play a pivotal role in managing chronic disease medication to reduce drug interaction risks. This study suggests developing a digital system for healthcare professionals to improve patient medication safety.

Keywords: Drug-drug interactions; polypharmacy; prolanis; type 2 diabetes.

INTRODUCTION

World Health Organization defines polypharmacy as the concurrent administration of five or more drugs[1], regardless of whether it is appropriate (evidence-based). In addition, Guillot et al.[2] gave a comprehensive overview of polypharmacy, including factors that affect it (from 4% to 57%) and factors that influence it, such as patient, disease, and healthcare system issues. The issue of polypharmacy has become a growing public health concern in all healthcare sector.[3,4] Moreover, the risks associated with polypharmacy are drug–drug interactions, higher risk of falls, adverse drug reactions, cognitive impairment, non-adherence, and poor nutritional status.[3,5,6] A cohort research in Korea found that polypharmacy was associated with a greater risk of hospitalization and death. Those risks negatively impact health outcomes and healthcare expenditures.[1,7] Then, the most vulnerable patient groups to the risk of polypharmacy tend to be elderly patients over

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65 and people living in nursing homes.[2,3,8] In chronic disease management today, polypharmacy becomes inevitable since the patients are treated for multiple diseases that required multiple prescriptions.[9,10]

Diabetes is a chronic disease that significantly impacts the lives of the patient themselves, families, and societies worldwide.[11] Due to its widespread occurrence, detrimental impacts on the economy and society, and decreased quality of the patients, diabetes mellitus ranks among the most dangerous chronic illnesses in the world.[12] In addition, environmental, lifestyle, and genetic variables have a role in aetiology and progression of diabetes itself.[13] Among the top 10 causes of death in adults, diabetes was estimated to have caused four million deaths worldwide in 2017.[11] Among three diabetes types, type 2 diabetes (T2D) accounts for approximately 90% of the total.[11] Moreover, type 2 diabetes mellitus dominantly causes most deaths in the world.[14] It is a result of relative insulin deficiency and peripheral insulin resistance.[12] The increase in type 2 diabetes is associated with obesity, hypertension, and aging population.[15] T2D affects an estimated 463 million adults aged 20-79, which is expected to reach 578 million by 2030.[13] With a prevalence of 10.8%, Indonesia is one of the top 10 countries with the highest prevalence of T2D.[13]

Patients with type 2 diabetes mellitus are initially treated with dietary modifications, increased physical activity and exercise, and weight loss.[16] Moreover, the incidence of type 2 diabetes in the elderly is 2.28 times higher, along with higher chance of geriatric syndromes called polypharmacy, which raises the possibility of drug interactions.[17] These lifestyle modifications are supported by diabetes self-management education and consultations with certified dietitians.[18]

When patients are diagnosed with diabetes, many medications could become appropriate therapy.[5] These include medications for dyslipidemia, hypertension, antiplatelet therapy, and glycemic control.[5] As a chronic condition, diabetes is associated with an increased risk of several other diseases due to its damage to macro and microvascular structures.[14,15] Moreover, diabetes-related macrovascular and microvascular complications cause the reduced quality of life, disability, and early death related to diabetes, including peripheral vascular disease, chronic renal disease, heart failure, coronary heart disease, cerebrovascular disease, diabetic retinopathy, and cardiovascular autonomic neuropathy.[18] In addition, several organs are negatively affected by diabetes, including brain, kidneys, heart, and eyes.[14,15] Furthermore, the risk of infection is also higher among diabetic patients. In addition to the risk of complications caused by diabetes, comorbidities also contribute to the numerous prescribed drugs.[17]

Through the government's National Health Insurance System (NHIS), Indonesia establish the Indonesian Chronic Disease Management Program (*Program Pengelolaan Penyakit Kronis /*PROLANIS) in 2014.[19] PROLANIS seeks to offer people with chronic illnesses, especially those with diabetes mellitus and hypertension, and a proactive approach to healthcare services. In addition, PROLANIS provide people with chronic illnesses with the best possible quality of life by providing cost-effective and efficient healthcare services. Moreover, PROLANIS offers exercise, education, home visits, health checks, and consultations. T2D has become the main focus of PROLANIS, besides hypertension.[18,19] This integrated health service program involves the patients, healthcare professionals, healthcare facilities, and NHIS. It aims to control clinical and laboratory outcomes, prevent disease complications, and improve patients' quality of life. Furthermore, monthly regular medical consultation meetings are become the routine activities in PROLANIS program.[18,19]

Previous studies have shown pharmacists' vital role in improving PROLANIS patients' outcomes.[20,21] In addition, a research suggests that pharmacists are essential in managing chronic diseases and optimizing the use of medications, including detecting drug interactions and educating patients.[21] Since the drug interactions can cause significant morbidity and mortality, minimizing the risk of drug interactions should be an objective of drug

therapy.[5,15] Generally, a drug interaction occurs when one or more medications are co-administered, resulting in their effectiveness or toxicity alterations.[5,15] pDDIs can occur regardless of whether they result in clinically meaningful consequences and essentially occur before actual DDIs.[22] Unfortunately, the research regarding the pDDIs among T2D patients in Indonesia are limited, especially for patients enrolled in PROLANIS Program. Therefore, this research aimed to identify the pDDIs of polypharmacy in T2D patients of PROLANIS Program. The findings in this research could provide insight into the frequency, severity, and risk factors associated with pDDIs in ambulatory T2D patients enrolled in PROLANIS Program.

METHODS

Study design, setting, and participants

This research was an observational and cross-sectional research. The researchers prospectively examined the prescriptions of 250 patients diagnosed with T2D in PROLANIS Program at Muhammadiyah Hospital, Bandung, who applied to outpatient clinic between April 1 and April 29, 2023. In addition, Muhammadiyah Hospital is a public secondary care hospital located in capital of West Java province, the most populous province in Indonesia. As PROLANIS Program requires monthly visits[19], the researchers assumed the data obtained during a given month would remain the same in the following months. The T2D patients aged 18 or more were selected consecutively. Then, the polypharmacy was defined as prescribing five or more drugs, and the prescriptions without polypharmacy were excluded from this research. The sample size for this research was determined using a total sampling method.

Ethical considerations

This research was approved by Research Ethics Committee of Universitas Padjadjaran (No. No.195/UN6.KEP/EC/2023). The individuals did not provide informed consent as the researchers studied the prescriptions.

Screening of potential drug-drug interactions

The researchers screened the drug-drug interactions using drugs.com and go.drugbank.com interaction checkers.[22–24] Specifically, these programs describe all potential drug interactions and indicate that the information is available on specific drugs within a given class. As well as revealing the clinical relevance of the interaction, the literature citations are provided for the interaction. Additionally, the programs provide recommendations on monitoring and managing medications. Moreover, the DDIs were also categorized according to severity level listed.

Statistical analysis

The researchers conducted descriptive analyses to determine the frequencies and percentages of categorical and mean ± standard deviation, median, and range of continuous data. Multicollinearity was checked to test correlation among independent variables using the variance inflation factor, and none was collinear. Moreover, bivariate logistic regression analysis was assessed to determine the association of each independent variable with the presence of pDDIs. Multivariable logistic regression was assessed to identify the predictors when all variables were included simultaneously. The researchers also conducted sub-analyses to determine the significant variables that associated with the number of pDDIs. In the analyses of pDDIs predictors, some variables' categories had to be merged due to small populations within each subset. All statistical analyses were performed in IBM SPSS Statistics version 22.0 (IBM Corp., New York, USA) with a significance level of $p<0.05$.

RESULTS

Characteristics of polypharmacy prescriptions

The researchers observed 250 polypharmacy prescriptions for T2D patients of PROLANIS Program. The mean age of patients was 62.70 ± 9.85 (range 24–92 years), and 155 (62%) were aged 60 or more (elderly patients). One hundred forty-two patients (56.8%) were females. The total number of drugs per prescription ranged

from 5 to 11, with a median of 6. Then, one hundred ninety-six prescriptions (78.4%) contained five to seven drugs in a single prescription. Polypharmacy prescriptions were most prevalent in Internal Medicine Department (92.8%) and Neurology Department (5.6%). Table 1 presents the characteristics of the studied prescriptions.

Table 1. Characteristics of studied prescriptions

Characteristics	*n* (%) / mean ± SD (range) / median (range)
Age (years)	62.70 ± 9.85 (24-92)
<60 (non-elderly)	89 (35.6)
≥60 (elderly)	155 (62.0)
Missing	6 (2.4)
Sex	
Male	94 (37.6)
Female	142 (56.8)
Missing	14 (5.6)
Number of drugs per prescription	6 (5-11)
5-7	196 (78.4)
8-10	52 (20.8)
>10	2 (0,8)
Prescriptions source (Department)	
Internal Medicine	232 (92.8)
Neurology	14 (5.6)
Cardiology	3 (1.2)
Endocrinology	1 (0.4)

Abbreviation: SD = standard deviation

Frequency and severity of potential drug-drug interactions

Figure 1 illustrates the number of pDDIs per polypharmacy prescription. It was found that pDDIs were present in 78.4% of prescriptions. A total of 515 pDDIs were identified, with a median of 2 pDDIs per patient (range 1–15).

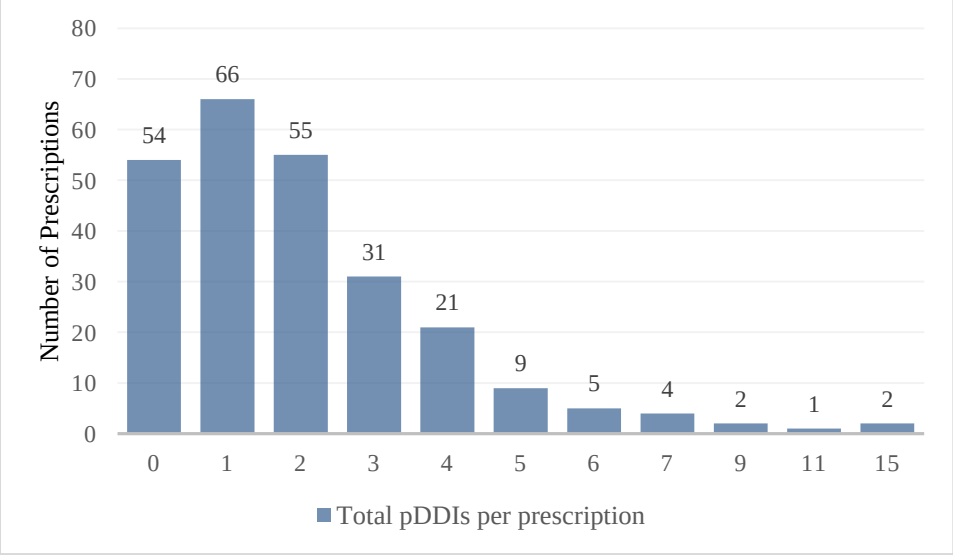

Figure 1. The number of pDDIs per polypharmacy prescription

Of the 515 pDDIs, 89.7% (*n* = 462) were of moderate severity, followed by 10.1% (*n* = 52) minor and 0.2% (*n* = 1) major pDDIs (Figure 2). In this research, it was found that 90 pairs of active substances interactions.

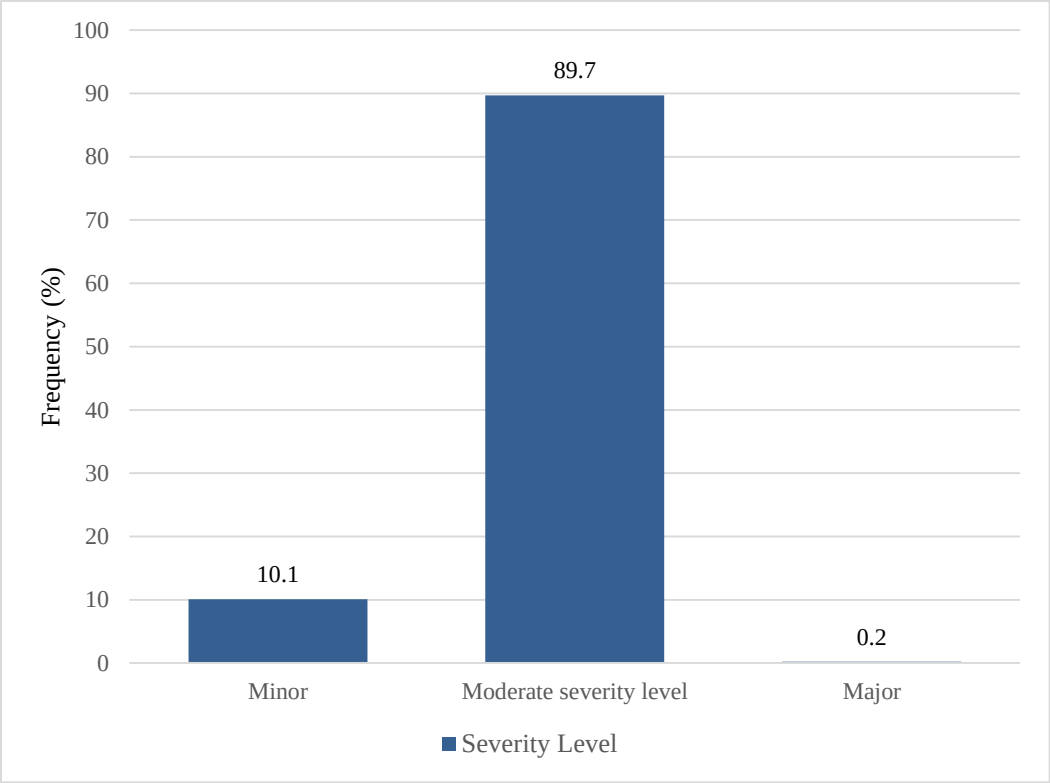

Figure 2. Severity levels of pDDIs identified

Table 2 shows the common drug pairs involved in pDDIs identified at each severity level. At the moderate severity level, most of the interactions were between glimepiride-metformin (18.6%), glimepiride-bisoprolol (8.3%), and metformin-ramipril (8.0%), whereas at the minor level, the most common interactions were between metformin-acarbose (6.6%). Then, the drug pair involved in pDDIs with major severity was pioglitazone + clopidogrel. Most drug pairs led to an increased risk of hypoglycemia (44.8%), whereas other possible consequences from the pDDIs identified were the reduced effects of antidiabetic agents (6.6%), hyperglycemia (3.3%), lactic acidosis (2.1%), and increased drug toxicity (1.7%).

Factors associated with the pDDIs

The researchers conducted a bivariate logistic regression analysis to compare the prescriptions with the presence and absence of pDDIS based on the patients' and prescriptions' characteristics. Accordingly, only prescription characteristics showed significant associations with the presence of pDDIs; *p*-values for the number of drugs per prescription and the prescription source variables were 0.004 and 0.019 respectively (Table 3).

Table 3. Bivariate and multivariable logistic regression results for predictors of the presence of pDDIs

Characteristics	Bivariate analysis			Multivariable analysis		
	SE	*p*-value	cOR (95%CI)	SE	*p*-value	aOR (95%CI)
Age (years)						
<60 (non-elderly) ≥60 (elderly)	0.33	0.453	1 0.78 (0.41-1.49)	0.36	0.278	1 0.27 (0.06-1.31)
Sex						
Male Female	0.32	0.074	1 1.78 (0.94-3.36)	0.34	0.120	1 1.69 (0.87-3.26)
Number of drugs per prescription						
5-7 ≥8	0.62	0.004*	1 5.98 (1.79-19.99)	0.75	0.007*	1 7.48 (1.73-32.32)
Prescriptions source (Department)						
Internal Medicine Others	0.50	0.019*	1 0.31 (0.12-0.83)	0.80	0.104	1 0.27 (0.06-1.31)

Abbreviation: SE = standard error; cOR = crude Odds Ratio; aOR= adjusted Odds Ratio; CI = Confidence Interval; *significant at *p*-value<0.05

However, according to multivariable analysis, only the number of drugs per prescription variable had a significant association (aOR = 7.48; 95% CI = 1.73-32.32) (Table 3). The researchers found that prescriptions containing eight or more drugs were 7.48 times more likely to have pDDIs than other polypharmacy prescriptions. In addition, table 4 presents the sub-analysis results in determining the number of drugs per prescription was associated with the number of pDDIs.

Table 4. The association between the number of drugs per prescription and the number of pDDIs

Characteristics	Binary logistic regression		
	SE	*p*-value	OR (95%CI)
Number of drugs per prescription			
5-7			1
≥8	0.57	0.010*	4.31 (1.42-13.11)

Abbreviation: SE = standard error; OR = Odds Ratio; CI = Confidence Interval;
*significant at *p*-value<0.05

The number of pDDIs was categorized into 1 = one to five interactions and 2 = more than five. Moreover, the result showed that prescriptions containing eight or more drugs were 4.31 times more likely to have more than five pDDIs (*p*=0.010).

DISCUSSION

The impact of one medication being altered (increased, decreased, or modified) by the presence of another medication when given consecutively is known as a drug-drug interaction (DDI).[25] As a subclass of adverse drug

events (ADEs), DDIs occur when two or more medications are taken at the same time and may result in a changed therapeutic effect.[26] In addition, the patients with diabetes are frequently treated with a combination of drugs due to the various comorbidities associated with the disease.[27] Identifying the frequency, severity, and risk factors associated with pDDIs among patients with T2D participating in the PROLANIS Program is essential. Besides assessing the safety of polypharmacy prescribing to these patients, it also provides suggestions for developing pharmacist services in the PROLANIS Program. An interprofessional care team made up of general practitioners, medical specialists, nurses, and pharmacists should be in charge of managing patients with chronic illnesses. In addition, pharmacists play a fundamental role in preventing, monitoring, and managing drug interactions, particularly in the case of chronic disease patients.[21,28] Pharmacists are also vital in educating patients, especially in raising awareness of the risks associated with their treatment.[21,27,29] Compared to doctors, pharmacists are able to give patients more precise advice when it comes to medication education. As a result, patients are more actively involved in achieving therapeutic goals and avoiding preventable fatal consequences, such as falls and hospitalizations.

This study found that pDDIs were present in 78.4% of the 250 polypharmacy prescriptions observed. It is higher than the frequency of pDDIs reported by other studies, either conducted in inpatient settings: Indonesia (57.7%); India (70%), or in outpatient settings: Indonesia (46.54%), Brazil (75%); Nepal (52.2%). Considering that outpatient settings are challenging to monitor compared to inpatient settings, the frequency of pDDIs identified in this setting raises some concerns. In addition, it is also needed to identify a total of 515 pDDIs, with a median of 2 pDDIs per patient. Furthermore, it is imperative that the severity of the pDDIs identified is evaluated since the presence of even one pDDI can lead to actual DDIs, thus decreasing the patient's quality of life and ultimately resulting in other harms.[22,30] These findings suggest that rational prescribing practices are of the utmost importance to chronic disease patients, especially diabetics.

Regarding severity, in the current study, approximately 89.7% (n = 462) of the pDDIs were of moderate severity, followed by 10.1% (n = 52) minor and 0.2% (n = 1) major severity. Linear with the previous studies, the most common severity of pDDIs in polypharmacy prescriptions for T2D patients was moderate level: Indonesia (89.39%)[31], Brazil (82,4%)[32], and Nepal (92.1%). The most frequent drug pairs associated with moderate-severity pDDIs were glimepiride-metformin, glimepiride-bisoprolol, and metformin-ramipril.

A combination of glimepiride and metformin is commonly used for patients with type 2 diabetes who have hypertension and dyslipidemia as main comorbidities and have micro- and macro-vascular complications.[33,34] However, a dose adjustment or blood glucose monitoring may be necessary due to the increased risk of hypoglycaemia.[35] Hypoglycemia can cause costly Emergency Room (ER) visits and hospital stays, as well as morbidity and mortality. It can also cause distress for people with diabetes and their families, medication no adherence, and disruptions to life and work.[36] Likewise, the sulfonylurea and beta-blocker combination was the most common drug-drug interaction in clinical routine.[35] According to Nurlaelah et al.[37], the drug pair interaction was also reported in their study of drug interactions in treating diabetes and hypertension in an outpatient setting in Indonesia. In addition, metformin and Angiotensin-converting enzyme (ACE) inhibitors are used together to treat hypertension in T2D patients. According to previous studies, this drug pair was also listed as a combination medication with potential drug-drug interactions. Because hyperglycemia increases platelet reactivity, clopidogrel becomes the cornerstone of ischemic secondary prevention in diabetic patients.[36] However, some observations implied that co-administering the two drugs might increase the risk of pioglitazone adverse reactions due to clopidogrel

inhibiting its metabolism.[38] A previous study in Indonesia did not find the major severity level of the pDDIs.[31] Several studies reported different drug pairs involved in major severity levels; however, clopidogrel was also present.[32,33]

Among diabetic patients, hypoglycemia is still one of the most frequent drug-related side effects and a major contributor to hospital admissions and ER visits.[39] Hypoglycemia is most common among older patients with multiple or advanced comorbidities, patients with long diabetes duration, or patients with a prior history of hypoglycemia. Moreover, insulin and sulfonylurea use, food insecurity, and fasting also increase the hypoglycemia risk.[40] In addition, most drug pairs (44.8%) increased the risk of hypoglycemia; as a result, dose adjustment, blood glucose monitoring, and improving patients' awareness of hypoglycemia symptoms were recommended.[41] In addition, patients may not recognize or receive treatment for hypoglycemia if they are unaware of its signs. Other possible consequences of the pDDIs identified, such as decreased effects of antidiabetic agents, hyperglycemia, lactic acidosis, and drug toxicity, require different management approaches. Still, in principle, the goal is to maintain normal blood glucose levels and improve patients' awareness of medication therapy risks.[6] It is essential that the patients recognize the signs and symptoms associated with possible adverse reactions, such as hypoglycemia, hyperglycemia, and lactic acidosis.[32,42] Particularly in diabetic populations worldwide, hyperglycaemic crises remain to be a significant contributor to morbidity and mortality.

In both bivariate and multivariable logistic regression analyses, the number of drugs per prescription had a significant association with the presence of pDDIs. Prescriptions containing ≥8 drugs were found to have a 7.48 times higher risk of pDDIs than those containing 5-7 drugs. Specifically, prescriptions containing ≥8 drugs were found to be 4.31 times more likely to have more than five pDDIs. It supports previous evidence of polypharmacy's role in pDDIs incidence and provides new insight into pDDIs among diabetics because it compares the risk of pDDIs among polypharmacy prescriptions.[22,32,33,43] There was no association between age, gender, or the source of prescriptions with the presence of pDDIs. Regarding the elderly and sex, the findings are consistent with those in the Brazilian study.[32] The two variables in the observed population may not affect the patients' disease-related conditions. About the prescription source variable, the significance in bivariate and insignificance in multivariable analysis indicates that the variable is only predictive due to its association with other predictors.[39]

The present study's results confirm the risk to the safety of T2D patients of the Prolanis Program subjected to polypharmacy. Nevertheless, polypharmacy prescribing is difficult to avoid due to the comorbidities of the aging population.[40] It becomes crucial to ensure rational or appropriate polypharmacy and monitor drug therapy in patients with T2D, especially in the PROLANIS Program.[40] Pharmacists play an essential role in reducing the risks of drug interactions by screening prescriptions before drug administration and providing drug information to patients to minimize these risks.[44] Due to the potential for toxicity, loss of efficacy, and adverse consequences, healthcare professionals such as prescribers, pharmacists, and nurses need to be adequately informed about drug-drug interactions.[45] A study found that patients receiving clinical pharmacy services from pharmacists were 11.6 times more likely to have controlled fasting blood glucose than those who did not receive such services.[44] Providing pharmacists with digital systems for detecting and preventing prescriptions with problems, as well as providing databases on pDDIs, interactions severity, and clinical recommendations, has become increasingly important today.[40] Moreover, mobile-enabled pharmacies are becoming more common, and digital transformation is crucial to integrating pharmacy data and providing personalized patient care.[46] It would decrease the risk of pDDIs and enhance the collaboration between health

professionals, particularly pharmacists and prescribers.

This is the first study assessing prospective prescriptions of diabetes patients enrolled in the PROLANIS Program. Additionally, the study provides information regarding the association between the number of prescribed drugs and the number of pDDIs. The study provides insight into medication safety among diabetics under the government's chronic disease management program. This study suggests developing a digital system for improving patient medication safety. Moreover, this study provides valuable data that will contribute to developing digital systems, especially for diabetics, a vulnerable group of patients due to the increased risk of complications, comorbidities, and the use of complex medications.

However, since this study was cross-sectional, the authors cannot infer a causal relationship among the factors of pDDIs. Secondly, the PDDIs in this study were determined by evaluating the medical prescriptions of ambulatory patients. Thus, the authors cannot assess their actual clinical impact, indicating that further research is needed in this area.

CONCLUSIONS

Potential drug-drug interactions (pDDIs) in patients with type 2 diabetes mellitus and cardiovascular diseases are common, with a prevalence of approximately 78.4% in polypharmacy prescriptions. The most prevalent pDDIs involve drug pairs like glimepiride - metformin, glimepiride - bisoprolol, and metformin - ramipril, which can increase the risk of hypoglycemia. Most diabetes patients were exposed to potential drug-drug interactions (pDDIs), with the number of drugs per prescription being its predictor. Most pDDIs identified were of moderate severity, involving the combination of glimepiride-metformin, glimepiride-bisoprolol, and metformin-ramipril, and most drug pairs interactions led to an increased risk of hypoglycemia. Given the complexity and risks associated with these interactions, the study suggests the development of a digital system for healthcare professionals to enhance medication safety by providing databases on interactions severity, clinical recommendations, and facilitating collaboration between healthcare providers. This digital system could assist in detecting and preventing pDDIs, ultimately improving patient outcomes and reducing the risks associated with polypharmacy prescriptions. To obtain a comprehensive understanding, further research should compare the potential and actual drug-drug interactions among the medication therapy of diabetes patients.

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تعدد الأدوية لدى مرضى السكري من النوع الثاني المشاركين في برنامج برولانيس في إندونيسيا: تحديد التفاعلات الدوائية المحتملة

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ملخص

يصبح تحديد التفاعلات الدوائية المحتملة أمرًا بالغ الأهمية في تقييم سلامة الأدوية بين مرضى السكري. هدفت هذه الدراسة إلى تحديد التفاعلات الدوائية المحتملة للتعدد الدوائي لدى مرضى السكري من النوع 2 (T2D) في برنامج إدارة الأمراض المزمنة أو برنامج إدارة مرض السكري المزمن .(PROLANIS) تم اختيار مرضى السكري من النوع 2 الذين تبلغ أعمارهم 18 عامًا أو أكثر على التوالي. تم تضمين عينة إجمالية من الوصفات الطبية التي تحتوي على 5 أدوية أو أكثر. كان متوسط عمر المرضى 62.70 ± 9.85 عامًا (المدى 24–92 عامًا)، وكان 62٪ منهم من كبار السن، و56.8٪ منهم من الإناث. كانت الوصفات الطبية للتعدد الدوائي أكثر انتشارًا في قسم الطب الباطني (92.8٪). من بين 250 وصفة طبية، احتوت حوالي 78.4٪ على تفاعل دوائي واحد على الأقل. تم تحديد ما مجموعه 515 تفاعل دوائي، بمتوسط تفاعلين دوائيين لكل مريض. ومن بين هذه الحالات، كانت 89.7% منها متوسطة الشدة. وكانت أزواج الأدوية المشاركة في pDDIs متوسطة الشدة هي جليمبيريد–ميتفورمين، جليمبيريد–بيسوبرولول، وميتفورمين–راميبريل. ويُعد عدد الأدوية لكل وصفة طبية مؤشرًا مهمًا لـ (aOR = 7.48 pDDIs؛ 95% .(CI = 1.73-32.32وكشف التحليل اللاحق أن الوصفات الطبية التي تحتوي على ثمانية أدوية أو أكثر كانت أكثر عرضة بنسبة 4.31 مرة لوجود أكثر من خمسة pDDIs (p = 0.010). يجب أن يلعب الصيادلة دورًا محوريًا في إدارة أدوية الأمراض المزمنة لتقليل مخاطر تفاعل الأدوية. تقترح هذه الدراسة تطوير نظام رقمي لمهنيي الرعاية الصحية لتحسين سلامة أدوية المرضى.

الكلمات الدالة: التفاعلات الدوائية؛ تعدد الأدوية؛ برولانيس؛ مرض السكري من النوع الثاني.

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Chemical Constituents and in Vitro/In Vivo Pharmacological Effects of *Mentha piperita* L. Essential oil in Different Regions of Algeria

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ABSTRACT

This study focuses on *Mentha piperita* L. cultivated in two regions with distinct bioclimatic levels. The aim is to assess and compare the yield, chemical composition, and pharmacological activities of the plant in these diverse environmental conditions. The phytochemical profile of *Mentha piperita* L essential oils was established using gas chromatographic analysis. The pharmacological activities were performed in vivo and in vitro using analgesic, anti-inflammatory, antibacterial and antioxidant assays. The optimal yield of essential oil is obtained from the region of Oued Souf with 1.02% and that of the region of Algiers is equal to 0.86%. The GC/MS analysis revealed a richness of the essential oil in Linalool as well as its derivatives linalyl acetate for Oued Souf and linalyl butyrate for Algiers. In all pharmacological activities, the essential oil of *Mentha piperita* L from Oued Souf region was significantly more potent than essential oil from Algiers. This study can contribute to the application of *Mentha piperita* L essential oil in the pharmaceutical industry as a promising natural reservoir of volatile compounds with noteworthy therapeutic properties.

Keywords: Anti-inflammatory, GC/MS, Lamiaceae, *Mentha piperita* ,phytochemical profile, linalool.

1. INTRODUCTION

In recent years, the exploration of natural remedies for various health conditions has gained considerable attention, prompting extensive research into traditional medicinal plants .These plants have been widely employed for addressing diverse ailments, with certain ones remaining integral to customary therapeutic practices for various health conditions [1]. Pain, inflammation, and bacterial infections remain pervasive challenges in healthcare, necessitating the quest for novel therapeutic agents with efficacy and without side effects [2]. Nowadays, advances in plant biochemistry have shown that plant species can synthesize thousands of different chemical constituents (phenolic compounds, alkaloids and terpenoids) [3, 4]. Essential oils possess a complex chemical composition; constituting a blend of diverse molecules, notably terpenes (non-aromatic hydrocarbons) and oxygenated compounds (alcohol, aldehyde, ketone, and ester)[5, 6].Various factors such as the harvesting time,

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cultivation location, plant part utilized, and production method are among the numerous variables influencing the chemical composition and quality of essential oils derived from distinct plant species [7]. Their pharmacological properties encompass stimulative, diuretic, analgesic, antiseptic, and antimicrobial properties [8, 9]. Lamiaceae family plants are abundant in essential oils, hold considerable importance in natural medicine, pharmacology, cosmetology, and aromatherapy [3]. Among these plants, *Mentha piperita* L that occupies a privileged place in digestive phytotherapy and used in traditional medicine for its antispasmodic, antimicrobial and antioxidant properties [3, 10]. Peppermint is perennial plant that grows to be 50-80 cm tall and the rhizomes are wide spreading, fleshy, and bear fibrous roots, whereas the leaves are dark green, and flowers are purple, it encompasses a persistent chalice surrounds the fruit divided into four parts, this type of mint would arise from a crossing between Mentha aquatic and *Mentha spicata*.

Currently, several varieties, descendants of this hybridization, are cultivated in the world. They are particularly prevalent in temperate and Mediterranean regions, in cool zones and in neutral environments [11, 12]. Although the genus is native of the Mediterranean region. It is cultivated all over the world for its flavor, its scent and as well as for the different medicinal and pharmaceutical applications. Peppermint oil is one of the most produced and consumed essential oils. The chemical composition of *Mentha piperita* varies greatly according to the soil and the harvesting time. Peppermint essential oil is mainly constituted of menthol (30% to 40%), menthone (20% to 65%), esters, coumarins and sulfur compounds [13, 14]. However, other authors have reported the existence of particular chemo-types like limonene (33.37%), and 1,8-Cineole (30.75%) chemo-types in Spain [15], and Terpinene chemo-type (19.7%) in Iran [16].

To the best of our knowledge, there is no scientific research that includes a phytochemical and pharmacological comparison of peppermint from various bioclimatic levels. Therefore, this work is interested to *Mentha piperita* L cultived in two regions located in different bio climatic levels in order to compare the yield, the chemical composition and pharmacological activities.

2. MATERIALS AND METHODS

2.1. Plant material

Mentha piperita L was collected in two different regions, a region with subhumid climate (Algiers), and in arid climate for the other (Oued Souf). The plants were collected in March 2022 from the two regions. The botanical identification of the plants was carried out at the National Institute of Agronomy, Algeria (INA) [17]. A standard voucher specimen is conserved in the herbarium of university of Florida (U.S.A) (voucher N◦:241569). The freshly collected plant at flowering stage, was dried in a dry and ventilated place, shielded from the light for 15 days, and then kept in bags. The aerial part (leaves, flowers and stems) was used for the extraction of the essential oil.

2.2. Microbial strains

The strains of the collections American Type Culture Collection (ATCC) were used to evaluate the antimicrobial potential of *Mentha piperita* L. essential oils, four bacterial strains (Staphylococcus aureus, (ATCC 6538), Pseudomonas aeruginosa, (ATCC 9027), Escherichia coli, (ATCC 4157), Bacillus subtilis, (ATCC 9372) and a yeast strain Candida albicans, (ATCC 24443) were tested. The strains were activated at 37°C for 24 hoursand at 25°C for 48 hours for the yeast [18].

2.3. Experimental animals

Adult male albino mice, Mus musculus, (average weight = 20±5 g) were obtained from the Pasteur Institute of Algeria. The animals were housed in standard laboratory conditions with a temperature of 24±2˚C and a 12-hour light/dark cycle. Animals received food and water ad libitum.

2.4. Essential oil extraction

The essential oil extraction from peppermint was carried out by Clevenger hydrodistillation [19]. A quantity of

100 grams of dried plant material from each plant was placed in a flask with 1 liter of water, equipped with a Clevenger apparatus and a condenser. The heating mantle was adjusted to a temperature of 90 °C. Distillation continued for 3 hours after the recovery of the first distillate drop. The obtained oils were preserved in an airtight brown glass bottle at 4 °C until utilization. The yield (R %) is defined as the ratio of the mass of essential oil (m HE) obtained to the mass of dry vegetable matter (m MVS) used, according to the following formula (1).

$$R\ (\%) = \frac{\text{m HE}}{\text{m MVS}} \times 100 \qquad (1)$$

2.5. Phytochemical analysis

A gas chromatographic analysis (HP Agilent 6890 Network GC System technology) coupled to a mass spectrometer (HP Agilent 5973 Network technology mass selective detector) was performed, in order to determine the different constituents of *Mentha piperita* L essential oils. Samples were separated on a capillary column HP-5MS (5% diphenyle and 95% dimethylpolysiloxane) 30m long and 0.32 mm diameter. The carrier gas is helium with a flow of 1 $mL.min^{-1}$. A volume of 0.5 µL of sample was injected in split mode with a ratio of 70/30. The oven temperature was programmed as follows: 60 °C for 8 minutes followed by an increase in temperature at a rate of 2 °C/min until 250 °C for 10 minutes. The temperature of the interface (GC-MS) was 280 °C and the temperature of the injector was 250 °C. The identification of the different constituents of *Mentha piperita L*. essential oil of was carried out based on mass spectra and retention indices (IR). They are calculated using a linear interpretation of the retention time of an alkane's series ranging from C8 to C22 [20].

2.6. Pharmacological activities

2.6.1 Antimicrobial activity

The qualitative evaluation of the antimicrobial activity was determined using disk diffusion method [21, 22]. Muller-Hinton's medium was used for bacteria and Sabouraud's for yeast. Sterile discs (MN 640w filter paper, Macherey-Nagel Gmbh and co, KG Germany), 9 mm in diameter were impregnated with 20µL of essential oil and then put down delicately on the agar seeded previously with a bacterial suspension of the five strains ATCC references adjusted to the 0.5 McFarland. Petri dishes were incubated at 37 °C for 24 hours for the bacteria and at 25 °C for 48 hours for the yeast [23]. The antimicrobial activity of the essential oil was determined by measuring the diameter of the inhibition zone (in mm) produced around the disks after incubation [24]. To determine the Minimum Inhibitory Concentration (MIC), cultures of bacteria or yeast (107-108CFU/mL) were carried out in the presence of decreasing concentrations (from 2% to 0.03%) of peppermint essential oil [25]. Dilutions of the essential oil were prepared in DMSO. The MIC was determined from Petri dishes where there is no visible culture [26].

2.6.2. Antioxidant activity

To measure the antioxidant activity of *Mentha piperita*, the DPPH test (diphenylpicrylhydrayl) was used according to the protocol described by Singh et al. (2015) [27]. In brief, 0.5 mL volumes of *Mentha piperita* L. at concentrations ranging from 10 to 50 µg/mL were combined with 1.5 mL of DPPH (0.004 g/L in methanol). Subsequently, the mixture underwent a 30-minute incubation in darkness at room temperature, following which absorbance was gauged at 517 nm against a methanol control using a Cecil CE2041 spectrophotometer from England. This process was iterated thrice for each concentration and sample. The Percentage of Inhibition (PI %) is determined using formula 2.

$$PI\ \% = (1 - \text{Sample})/(\text{Blank}) \times 100 \qquad (2)$$

A positive control is prepared using a solution of Butylated hydroxytoluene (BHT) as standard antioxidant. The variation of the reducing power according to the concentration of HE and BHT allows us to calculate the Inhibitory Concentration IC_{50} [28]. The extract concentration required to scavenge 50% of the DPPH radical is called IC_{50} and lower IC_{50} values indicate higher antiradical activity.

2.6.3. In-Vivo pharmacological activities

Analgesic activity

The analgesic potential of *Mentha piperita* L. essential oils was evaluated using acetic acid induced writhing test described by [29]. 6 groups each comprised of 6 mice (n=6) were used in this investigation. 4 groups received an oral dose of 0.5 mL of essential oils respectively at doses of 100 and 500 mg/kg per body weight. 2 other groups used as positive and negative controls, were treated respectively with paracetamol® at 500 mg/Kg b.w and saline solution. 30 minutes after treatment, animals were intraperitoneal injected with 0.5 mL of 0.6% acid acetic to induce writhes, then the count of abdominal contractions during 15 min. The protection percentage against abdominal writhes was used to assess the analgesic effect and was calculated as in formula 3.

Protection (%) = [(Wt -We/Wt)] 100 (3)

In which, We represents the mean value of writhes in treated groups and Wt represents the mean value of writhes in control groups.

- *Anti-inflammatory activity*

Carrageenan induced paw edema model was used to assessed the anti-inflammatory activity of *Mentha piperita* L. essential oils according to [30]. Mice were divided into 6 groups of six animals. One group served as negative control received orally 0.5 mL of saline solution, 4 groups were treated with *Mentha piperita* L. essential oils at doses of 100 and 500 mg/kg b.w. While, the reference group received 500 mg/kg b.w of Diclofenac. A volume of 0.05 mL of 1% carrageenan freshly prepared in saline solution was injected intradermal into the plantar side of the right hind paw of the mice one hour after the oral route of treatments. 4 hours after carrageenan injection, animals were sacrificed by cervical dislocation and the two posterior legs were cut at tarsal joint, then immediately weighed.

The anti-inflammatory activity was calculated as percentage inhibition of edema following the 4.

Inhibition% = [(PEC - PET)/PEC] 100 (4)

where, % PEC is the percentage edema of the control (negative control) and % PET percentage edema of the test (diclofenac and *Mentha piperita* L. essential oils). The percentage of edema is calculated using the formula 5.

PE (%) = [M(RPW)-M(LPW)]/M(LPW) 100 (5)

In which, M(RPW) is the mean weight of the right paw per group, and M(LPW) is the mean weight of the left paw per group.

2.7. Statistical Analysis

The results were presented as the mean ± standard deviation (SD). To assess significant differences among the studied samples, an analysis of variance (ANOVA) test followed by Tukey's multiple comparison test was conducted using Statistica version 6.0 statistical software. The values $P<0.05$ were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Yields of essential oils

The results obtained showed that the yields of essential oil extracted by hydrodistillation average is 1.02±0.01% for mint extracts from Oued Souf region and 0.82±0.01% for those of Algiers region (Table 1).

Table 1. Yields percentage (%) of *Mentha piperita* L. essential oils from Algiers and Oued Souf regions.

Oued Souf region	Algiers region	AFNOR standars
1.02±0.01	0.82±0.01	0.38 - 1.20

All values are represented as mean ± SD of three measurements

As shown in Table 1, the extraction yield of *Mentha piperita* L. essential oil from Oued Souf region is higher than those from Algiers region. These yields are within the range allowed by the AFNOR standards. The current investigation centered on exploring the phytochemical diversity and biological properties of *Mentha piperita* L.

essential oils cultivated in Algeria. The yield of *Mentha piperita* L. essential oil is different depending on the harvest region, that of Oued Souf has a higher yield than that of Algiers. Generally, the essential oil yield depends usually on the soil properties, the harvest period and finally the extraction techniques [31]. Our yields are higher than those obtained by *Mentha piperita* L. essential oils of Benin, estimated at 0.45±0.02% [32] and in Russia with a yield of 0.34% [33].

3.2. Phytochemical analysis

Chemical composition of peppermint essential oil from Algiers and Oued Souf regions were established using gas chromatography coupled to mass spectrometry allowed us to identify 99.10% of the constituents for mint essential oil grown in Algiers and 98.46% of the constituents of the essential oil of that grown at Oued Souf (Table 2). A high proportion of linalool and its acetate and butyrate derivatives (Table 2; Figure 1) characterized both essential oils. Linalool is the major constituent of peppermint from both sources with respectively 34.66 and 25.36% for Algiers and Oued Souf. On the other hand, linalool derivatives are characteristic of the study regions, linalyl butyrate (26.87%) is characteristic of the essential oil of the Algiers region, whereas Oued Souf's one is characterized by linalyl acetate (22.04%).

The chromatograms of peppermint essential oil from Algiers and Oued Souf regions are respresented in Figure 1.

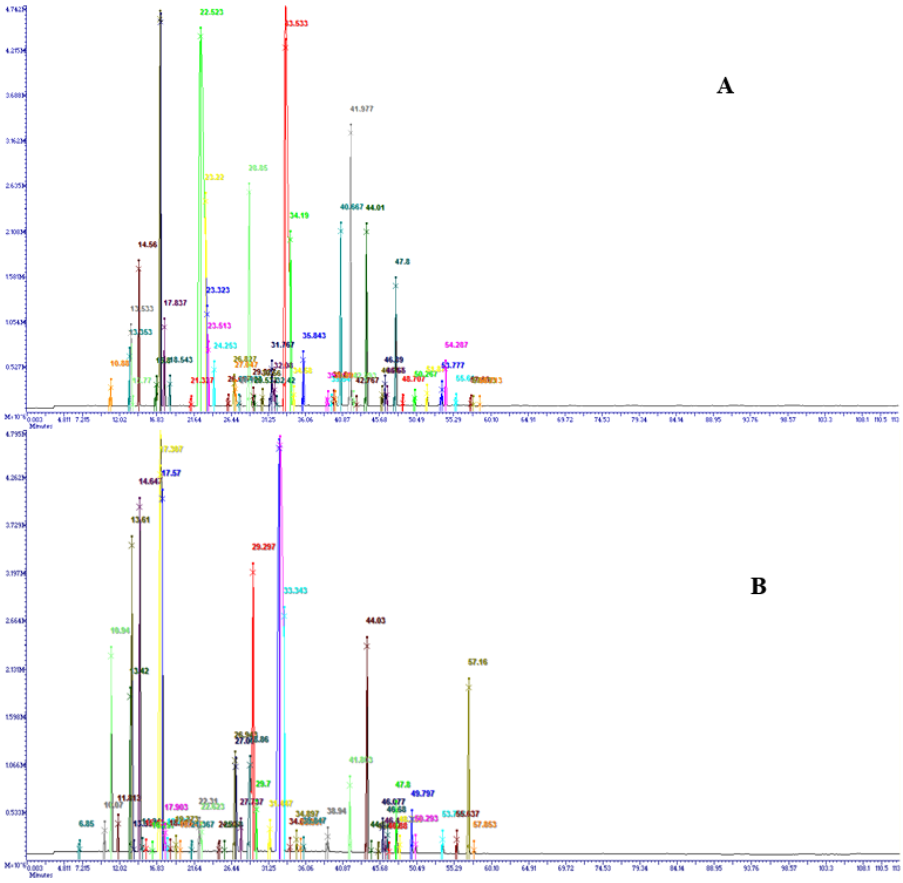

Figure 1. GC-MS chromatograms of *Mentha piperita* L. essential oils (A- Algeriers region, B- Oued Souf region).

Table 2. Chemical composition of the essential oil of peppermint from two different regions.

IR	Compounds	Relative percentage (%)	
		Algiers region	**Oued Souf region**
1004	Alpha pinene	-	*0,30*
1008	alpha- Thujene	0,19	-
1014	Sabinene	-	0,13
1015	Beta pinene	-	0,15
1018	alpha-phellandrene	0,57	-
1019	beta- Thujene	0,857	-
1019	Beta- Myrcene	-	0,97
1023	alpha –Myrcene	1,64	-
1029	Para –Cymene	-	0,22
1032	o-cymene	0,3	-
1034	Eucalyptol	9,89	3,1
1037	trans -alpha-ocimene	0,88	3.47
1038	Gamma terpinene	-	0.27
1040	Beta-cis-ocimene	0,22	1.27
1047	Alpha-terpinolene	-	0.16
1056	Linalool	34,66	25.36
1059	n-Amylisovalerate	0,828	-
1060	1-Octen-1-ol, acetate	0,52	0.35
1063	3-Octanol, acetate	0.375	-
1073	Isomenthone	0,31	-
1074	4-Octene-2,7-diol, 2,7-dimethyl-, Z-	0.267	-
1082	alpha-Terpineol	5.903	8.05
1084	3,7-Octadiene-2,6-diol, 2,6-dimethyl-	0.148	-
1094	cis-geraniol	0.708	-
1095	cis-isopulegone	0.338	-
1099	Linalyl acetate	-	22.04
1099	Geraniol	-	6.54
1101	linalyl butyrate	26.873	-
1102	linalyl formate	0,63	-
1197	Nerylacetate	-	2.4
1223	2,6-Octadien-1-ol, 3,7-dimethyl-, acetat	2.93	0.33
1259	p-Mentha-1,45 ,8-triene	-	0.98
1269	Geranylacetate	4.605	4.46
1282	alpha.-Gurjunene	-	0.34
1500	Trans-caryophyllene	2.595	2.2
1514	trans-.beta.-Farnesene	-	0.35
1519	Alpha-Humulene	0.125	0.19
1522	Germacrene D	0.235	1.84
1535	cis-muurola-4(14),5-diene	1,74	-
1549	Elemol	-	5.62
1561	Viridiflorol	0.43	1.50
1573	Alpha- selinene	0.131	1.57
1581	Alpha –eudesmol	-	3.16
1588	Valencene	-	0.86
1590	Trans cirtal	0.207	0.28

The phytochemical analysis revelead that the composition of monoterpenes and sesquiterpenes of peppermint grown in Algeria from Algiers and oued souf regions differs from that grown in France, in India and in Italy by the presence of linalool and its derivatives and the absence of menthol and its derivatives [34, 35].This characteristic composition could allow us to consider that the peppermint cultivated in the two regions (North and South) of Algeria and Morocco [36], would be a new chemotype for Algeria with linalool and its derivatives as the majority compound. The studies carried out on the essential oil of *Mentha piperita* L. showed a great diversity in its chemical composition. Indeed, the existence of specific chemotypes has been demonstrated in Spain, chemotype of limonene (33.37%), and 1.8-Cineole (30.75%) [19], and in Iran, chemotype of Terpinene (19.7%) and Pipertitinone oxide (19.3%) [10]. The essential oil of peppermint grown in Morocco, Iran**,** ~~in~~ Russia and in the Himalayas is consisting essentially of menthol and its derivatives, menthyl acetate, menthone, and menthofurane [34, 37, 38]. Thus, these different chemotypes of the essential oil of *Mentha piperita* L may be due to ecological factors and the difference in geographical positions [35, 39].

3.3. Antimicrobial activity

The antimicrobial activity of the essential oils was determined by measuring the inhibition zone diameters surrounding the absorbent discs after incubation under suitable conditions for the tested germ development (Table 3).

Table 3. Antimicrobial activity of peppermint essential oil on a variety of microbial strains.

			Inhibition diameter (mm)	
		Strains	**Algiers region**	**Oued Souf region**
Bacteria	Gram +	*Bacillus subtilis*	20.33±0.,57	22.00±0.50
		Staphyloccocus aureus	18.66±0.57	20.83±1.75
	Gram -	*Escherichia coli*	13.83±0.28	15.83±0.76
		Pseudomonas aeruginosa	9.16±0.50	9.50±0.28
Yeast		*Candida albicans*	48.16±0,28	50.33±1.15

All values are represented as mean ± SD of three measurements.

The findings obtained indicated that the essential oil of *Mentha piperita* L. from the Oued Souf region is more effective than that from the Algiers region against all strains tested.

As per the Chifundera *et al.* (1990) [40], the essential oil of *Mentha piperita* L. exhibits Strong effect against Gram+ bacteria (*Bacillus subtilis* and *Staphyloccocus aureus*) whose inhibition zones ranged from 18.66±0.57 to 22.00±0.50 and a moderate inhibitory activity against Gram- bacteria : *Escherichia-coli* with inhibition zones of about 13.83±0.28 and 15.83±0.76 ,respectively. For Oued Souf and Algiers regions, while showing no inhibitory effect against *Pseudomonas aeruginosa* (Table 3). *Candida albicans* strain showed high sensitivity to *Mentha piperita* L. essential oil for both samples with inhibition zones of 48.16±0.28 mm (Algiers region) and 50.33±1.15 mm (Oued Souf region). Inhibition zone diameter of yeast is much larger than that observed for bacteria. Therefore, the antifungal activity of *Mentha piperita* L. essential oil is more important than the antibacterial activity.

3.3.1. Quantitative determination (MIC)

The MIC results show that the five strains are sensitive to different concentrations of *Mentha piperita* essential oil (Table 4), with the exception of Pseudomonas aeruginosa from Algiers region, which shows resistance. The MIC values obtained are quite low; they range within 0.125 and 0.03%.We found that the essential oil of *Mentha piperita* from Oued Souf region has a MIC of 0.25% for Gram + bacteria (Bacillus subtilis and Staphylococcus aureus), and a MIC of 0.5% for Escherichia coli, 0.125% for Pseudomonas aeruginosa and a MIC < 0.03% for Candida albicans.

Table 4. MIC values of Peppermint essential oil from two regions (+: Growth (resistant strain).-: Inhibition (sensitive strain).

Concentrations			Regions	2.00%	1.0%	0.5%	0.2%	0.12%	0.06%	0.03%
Bacteria	Gram+	*Bacillus subtilis*	Algiers	-	-	-	-	-	-	+
			Oued Souf	-	-	-	-	+	+	+
		Staphylococus aureus	Algiers	-	-	-	-	-	-	+
			Oued Souf	-	-	-	-	+	+	+
	Gram-	*Escherichia coli*	Algiers	-	-	-	-	+	+	+
			Oued Souf	-	-	-	+	+	+	+
		Pseudomonas aeruginosa	Algiers	+	+	+	+	+	+	+
			Oued Souf	-	-	-	-	-	+	+
Yeast		*Candida albicans*	Algiers	-	-	-	-	-	-	+
			Oued Souf	-	-	-	-	-	-	(≤0.03)

The antimicrobial activity was assessed using disk diffusion method which revealed the efficacies of *Mentha piperita* L. essential oil from Oued Souf region is more effective than that from the Algiers region against all strains tested. The antimicrobial effect of medicinal plants is mainly associated to the presence of secondary metabolites [41]. The action mode of the essential oils depends on the active components type and characteristics, especially their hydrophobic property which allows them to penetrate the double layer of phospholipids of the bacterial cell membrane. This can induce a change in the fluidity of the membrane, a chemo-osmotic disturbance and a leakage of ions (potassium ion) [42]. The obtained findings of this activity can be attributed to its richness in oxygenated monoterpenes (linalool, alpha-terpineol, eucalyptol), which has shown its power to fight against many bacterial strains tested, despite the presence of a low concentration of alpha-terpineol and eucalyptol. these compounds being known for their antibacterial activity [43]. Indeed, these compounds lead to lesions in the microorganism's cell membrane, increasing cell permeability and consequently a loss of cellular constituents and thus the death of the bacteria [44]. Phenols (thymol, carvacrol and eugenol), alcohols, (α-terpineol, terpinen-4-ol, linalool), aldehydes, ketones and more rarely carbides are chemical compounds known for their antimicrobial efficacy and broad spectrum [45]. Alcohols are generally better known for both their bacteriostatic action on vegetative cells, through protein denaturation. Aldehydes, which are strongly electronegative, are powerful antimicrobial agents when reacting with the vital nitro compounds (proteins and nucleic acids) of bacteria [46].

3.4. Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl DPPH method was used to assess the antioxidant capacity of peppermint essential oils. The averages of percentage inhibition (Pi) and IC_{50} values for peppermint essential oils and BHT as a function of concentration are listed in Table 5.

Table 5. Inhibition percentages and IC_{50} values for essential oils and BHT. All values are represented as mean ± SEM of three measurements; [a] $P < 0.05$ compared with reference.

Concentration µg/mL	10	20	40	60	100	150	IC_{50}
Pi%(HE) Oued Souf	46.51±0.51	50.66±0.57	54.25±1.09	57.50±0.50	67.52±0.50	74.73±0.46	23.33±1.20[a]
Pi%(HE) Algiers	18.64±1.19	23.46±1.34	38.49±1.47	65.72±2.57	86.52±2.52	89.55± 0.7	38.37±0.88[a]
Pi%(BHT)	36.09±5.24	47.21±4.41	62.10±6.47	72.33±8.45	85.13±3.42	92.08±2.86	21.79±2.62

The essential oil from Oued Souf region has a strong significantly ($P<0.05$) antioxidant activity at low concentration (46.51%) than the one from Algiers region and particularly compared to BHT; an increase inantioxidant activity is observed according to the concentration. However, the antioxidant activity of the Oued Souf sample is significantly ($P<0.05$) higher than that of Algiers. The antiradical activities obtained show that essential oils have a moderate antioxidant activity with IC_{50} values of 23.33±1.02, and 38.37±0. 88µg/mL for the peppermint essential oils of Oued Souf and Algiers respectively, while the IC_{50} value of the standard antioxidant BHT was 21.79±2.62 mg/mL close to that of IC_{50} of Oued Souf (23.33±1.02 µg/mL) (Table 5).

The antioxidant potential of *Mentha piperita* L. essential oils is probably related to the presence of alcohol, linalool in both samples, and to other oxygenated substances such as alpha terpineol and eucalyptol. Monoterpenes and oxygenated sesquiterpenes contribute redox characteristics to essential oils, leading to their antioxidant potential [47]. The essential oils of peppermint from Turkey (menthol (38.06%)), Morocco (menthone (29.01%)), and Egypt (Menthol (40.47%)) have an anti-free radical capacity much less important than that of our extracts composed mainly of linolool and its derivatives [7, 48].

3.5. In-vivo pharmacological activities

➢ *Analgesic activity*

The acetic acid induced writhing test was used to evaluate the analgesic potential of *Mentha piperita* L. essential oils. The obtained findings on the acetic acid induced abdominal constrictions tested in mice is showed in Figure 2.

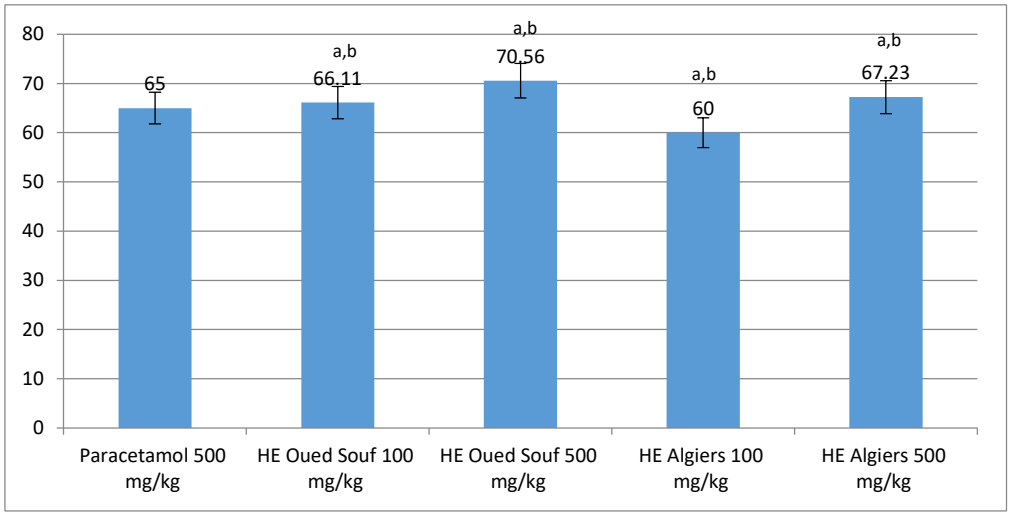

Figure 2. Analgesic effect of *Mentha piperita* L. essential oils and Paracetamol® using acetic acid induced writhing test.

The results revealed that the oral intake of *Mentha piperita* L. essential oil from Algiers and Oued Souf regions led to a significant ($P < 0.05$) reduction in the number of writhing in a dose-dependent manner (Figure 2). *Mentha piperita* L. essential oil obtained from the Oued Souf region presented the highest analgesic potential with significantly ($P<0.05$) inhibition percentages of 66.11%±1.50 and 70.56%±2.08 respectively at 100 and 500 mg/kg doses. This analgesic effect is greater than that of paracetamol 500 mg/kg (65.00%±2.00). The essential oil of *Mentha piperita* L. from Algiers at dose of 500 mg/kg exhibited analgesic action superior than paracetamol with 67.23%±1.52 inhibition.

➢ *Anti-inflammatory activity*

The results demonstrating the anti-inflammatory activity of *Mentha piperita* L. essential oils, as assessed through the carrageenan-induced hind paw edema test, are presented in Table 6.

Table 6. Anti-inflammatory activity of *Mentha piperita* L. essential oils on carrageenan induced paw oedema in mice.

Groups	Dose (mg/kg b.w)	Oedema percentage (%)	Inhibition p ercentage (%)
Control (saline)	---	46.08±1.93	---
Diclofenac®	100	12.98±1.72[a]	71.83±1.16[a]
***Mentha piperita* L Algiers**	100	18.07±0.97[a,b]	60.78±0.81 [a,b]
	500	15.51±1.23 [a,b]	66.34±1.19 [a,b]
***Mentha piperita* L Oued Souf**	100	16.03±1.13 [a,b]	65.21±0.74 [a,b]
	500	13.17±0.21 [a,b]	71.41±1.05 [a,b]

All values are represented as mean ± SEM of six measurements; Tukey test:a $P < 0.05$ compared with control; b $P < 0.05$ compared with Diclofenac®.

As depicted in Table 6, the essential oils of *Mentha piperita* L., along with Diclofenac®, demonstrated a significant reduction ($P<0.05$) in paw edema inflammation compared to the control. The *Mentha piperita* L. essential oil from the Oued Souf region exhibited the highest dose-dependent anti-inflammatory potential, ranging from 65.21% at 100 mg/kg to 71.41% at a 500 mg/kg dose. This anti-inflammatory efficacy was equal to the reference drug Diclofenac® (71.83%). The administration of *Mentha piperita* L. essential oil from Algiers region at doses of 100 and 500 mg/kg, exhibited a decreased inhibition percentage of paw edema compared to *Mentha piperita* L. from the Oued Souf region and the reference drug (with percentages of 60.78% and 66.34%, respectively).

Similar results were observed in analgesic and anti-inflammatory activities, the essential oil of *Mentha piperita* L. from the Oued Souf region was the most active. These results suggest the existence of active substances on the mediators common to inflammation and the painful process, such as histamine and prostaglandins. Terpenoids are recognized for their inflammatory effects [49, 50]. These molecules probably act as non-steroidal anti-inflammatories by inhibiting the enzymatic activity of cyclooxygenase and thus limiting the quantity of pro-inflammatory mediators produced during this process [41, 51, 52]. The exclusive presence of monoterpenes as Beta pinene, sabinene, Beta- Myrcene and sesquiterpenes such as Elemol and Alpha–eudesmol in the essential oil of *Mentha piperita* L. from Oued Souf region can explain its analgesic and anti-inflammatory potentialities exceeding that of Algiers region [53-55].

4. CONCLUSION

This is the first work about Phytochemical composition and pharmacological activities of *Mentha piperita* L essential oil cultivated in two different regions in Algeria. It has been demonstrated that ecological factors and geographical positions can affect the yeilds, phytochemical content and pharmacological properties. The essential oil of *Mentha piperita* L. growing in Oued Souf region is more efficacy in pharmacological activities. Thus, this study may contribute to the potential use of *Mentha piperita* L. essential oil in the pharmaceutical industry as a promising natural reservoir of bioactive compounds possessing noteworthy therapeutic properties.

Ethical approval

The experimental procedures were approved by the Ethical Committee of Animal Experimentation (CEEA) of University of Sciences and Technology Houari Boumediene (USTHB) with approved Ref N°: CEEA-USTHB-08-2023/11118, Algeria.

Conflict of interest: The authors declare no conflicts of interest.

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المكونات الكيميائية والتأثيرات الدوائية في المختبر/في الجسم الحي لزيت النعناع الفلفلي من نوع *Mentha piperita* L. في مناطق مختلفة من الجزائر

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ملخص

تتناول هذه الدراسة النعناع الفلفلي *Mentha piperita L.* المزروعة في منطقتين تتمتعان بمستويات بيئية مناخية مختلفة. الهدف هو تقييم ومقارنة الإنتاج والتركيب الكيميائي والأنشطة الدوائية للنبات في هذه الظروف البيئية المتنوعة . تم تحديد الملف الكيميائي النباتي لزيوت النعناع الفلفلي باستخدام تحليل الكروماتوغرافيا الغازية. تم إجراء الأنشطة الدوائية في المختبر وفي الجسم الحي باستخدام اختبارات مسكنة، ومضادة للالتهابات، ومضادة للبكتيريا، ومضادة للأكسدة. تم الحصول على أعلى إنتاجية من الزيت العطري من منطقة وادي سوف بنسبة 1.02%، بينما كان إنتاج منطقة الجزائر 0.86%. كشفت تحليلات GC/MS عن غنى الزيت العطري بالينالول وكذلك مشتقاته مثل أسيتات الينالیل في وادي سوف وبوتيرات الينالیل في الجزائر. في جميع الأنشطة الدوائية، كان زيت النعناع الفلفلي من منطقة وادي سوف أكثر فعالية بشكل ملحوظ من الزيت العطري من الجزائر. يمكن أن تساهم هذه الدراسة في تطبيق زيت النعناع الفلفلي في صناعة الأدوية كمصدر طبيعي واعد للمركبات المتطايرة ذات الخصائص العلاجية البارزة.

الكلمات الدالة: مضاد للالتهابات، كروماتوغرافيا الغاز/مطياف الكتلة، الشفوية (أو الفصيلة الشفوية)، النعناع الفلفلي، الملف الكيميائي النباتي، الينالول.

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Vitamin A Precursor: Beta-Carotene Alleviates the Streptozotocin-Induced Diabetic Retinopathy in Male Adult Zebrafish via the Regulation of the Polyol Pathway

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ABSTRACT

Diabetic retinopathy (DR) is a progressive neurovascular disorder due to damage to retinal blood vessels. Beta-carotene acts as retinal chromophores and initiates photo-transduction and epithelial maintenance. Beta-carotene (BC) is present in palm oil mill effluent, and it is called palm oil mill effluent-derived beta-carotene (PBC). The present study is designed to evaluate the effect of PBC in streptozotocin-induced DR in zebrafish by measuring the oxidative stress, inflammation, and polyol pathway markers. The five groups of healthy *Danio rerio* were used in this study. The diabetes retinopathy was instigated by intraperitoneal administration of streptozotocin (STZ) followed by intravitreal administration of STZ on the 7th day. The exposure of PBC (50 and 100 mg/L) and dexamethasone (DEX) was administered for 21 continuous days. The DR-associated visual behaviours *i.e.*, optomotor response (OMR) and startle response (SR) were appraised on 0, 7, 14, and 21st days. The biochemical changes *i.e.*, plasma glucose & homocysteine (HCY); retinal tissue lipid peroxidation, reduced glutathione (GSH), tumor necrosis factor-alpha (TNF-α), superoxide dismutase (SOD) and total protein levels were estimated. The lens was used for the evaluation of polyol pathway markers *i.e.*, sorbitol dehydrogenase (SDH) and aldose reductase (AR) activity. The PBC potentially attenuated the DR with the regulation of biochemical abnormalities which is similar to DEX treated group. Hence, PBC can be used for the management of DR due to its anti-hyperglycemia, antioxidant, anti-inflammatory, and polyol pathway regulatory actions.

Keywords: Aldose reductase; free radical; intravitreal injection; optomotor response; palm oil mill effluent; sorbitol dehydrogenase.

1. INTRODUCTION

Retinopathy is a disease caused by damage to the retina and it involves abnormal vascular tissue growth in the retina [1]. The progression of retinopathy shows reversible blindness with retinal blood vessel damage and light sensation to the retina [2]. In diabetic conditions, it rapidly causes retinal barrier dysfunctions triggered by the accretion of advanced glycation end products (AGE) products [3]. Clinically, diabetic retinopathy (DR) showed exudates, intra-retinal hemorrhage, micro-aneurysms, macular edema, macular ischemia, and the appearance of cotton-wool spots in the ocular (retinal) region [4,5].

Vitamin A is an essential nutrient for vision function and it reaches the retinal layer with the highest concentration (3 millimolars) [6]. Primarily retinoids and pro-vitamin carotenoids act as pro-vitamin A, retinol, and retinoic acid and they support the neurosensory functions of the retina

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[7]. The 11-cis-retinal form is mostly found in the retina and it is a crucial chromophore for all photoreceptor cells. These are also bound in the form of a Schiff base with rhodopsin in rod cells and photopsins in cone cells [8]. The deficiency of vitamin A is known to enhance retinopathy progression, especially in diabetic conditions [9]. Experimentally, the administration of vitamin A is reported to decrease the possibility of DR progression via control of multiple biochemical and molecular pathways [10].

The carotenoids *i.e.,* BC are a vitamin A precursor that acts on retinal chromophores and initiates photo-transduction, & maintains the retinal epithelial barrier functions [11]. A large quantity of beta carotene is present in carrots, tomatoes, spinach, sweet potatoes, broccoli, and winter squash including palm oil and their waste (palm oil mill effluent; POME) [12,13]. It produces therapeutic actions via free radical scavenging, anti-inflammatory, immune cell function, and regulation of vascular barrier functions [14]. Streptozotocin (STZ) is a natural alkylating agent (glucosamine-nitrosourea compound) and it induces diabetes mellitus and its complications via carbonium ions associated deoxyribonucleic acid (DNA) damages of islet β cells and other localized cells [15]. In experimental animals, DR is commonly produced by the administration of STZ [16]. The pathogenesis of DR is mostly caused by the development of retinal oxidative stress, inflammation, vascular dysfunction, accumulation of AGE products, and alteration of polyol pathways which leads to visual and cognitive dysfunctions [17–19]. The zebrafish is the finest model for assessment of diabetes mellitus and retinal & visual functions [20–22]. Zebrafish possess various significant advantages over mammalian animal models like rats and mice due to their rapid growth, easy handling, and clear visual functions [23]. Furthermore, the phenotypes of zebrafish closely resemble animal models and also mimic human diabetic microvascular complications like retinopathy [24]. Besides, the DR-associated behavioural assessment is easier in zebrafish than in rodents [25,26]. Moreover, beta carotene potentially attenuates diabetes-associated neuropathic pain in female zebrafish via inhibition of matrix metalloprotease-13 action [27]. The manifestation of diabetic complications like retinopathy is varied in male rats than female rats. The rate of diabetic complications development in female rats is only 30% [28]. However, the gender basis effects in the progression of DR in zebrafish models are not clear [29]. Generally, the female reproductive hormone i.e., estrogen possesses the potential neurovascular and retinal tissue-protective actions whereas testosterone induces the risk of chorioretinopathy [30]. Therefore, the current research work was planned to perform the vitamin A precursor *i.e.,* PBC action in STZ-induced DR in male adult zebrafish and the regulation of oxidative, inflammatory, and polyol pathways.

2. MATERIALS AND METHODS

2.1 Animals

The 8-month-old wild kind of male-grown zebrafish (**Danio rerio**) was utilized. Zebrafish were kept in 10 L of consumable water (in a fish tank). The fish tank contains a stone bubble aerator for the enrichment of dissolved oxygen in water; a glass thermometer for maintaining water temperature (26 ± 1 °C); and a light-emitting diode (LED) with a timer switch for providing the 14 hours light & 10 hours dark cycles environment. This study was supported by the Institutional Animal Ethics Committee (AUAEC/FOM/2022/01) and animal care was followed as per the rules of the Institutional Animal Ethics Committee. Animals were quarantined for 14 days before the initiation of the experiment. The neurobehavioural monitoring **i.e.,** optokinetic motor response (OMR) and startle response (SR) were evaluated as shown in the experiment protocol.

2.2 Drugs and chemicals

1,1,3,3-Tetra methoxy propane, 5,5-dithibis(2-nitrobenzoic acid), reduced glutathione, thiobarbituric acid, and streptozotocin (STZ) were purchased from Merck - Life Science, Darmstadt, Germany. PBC was

isolated as described in our previous article [13]. The positive control drug *i.e.,* dexamethasone sodium phosphate vial was purchased from Mylan Pharmaceutical Industry Company, Pennsylvania, United States.

2.3 Induction of diabetic retinopathy (DR)

The diabetes mellitus was initiated by the application of streptozotocin (STZ; 350 mg/kg) as portrayed by Olivares *et al.* [31] with minor changes of Wang *et al.* [32]. Briefly, zebrafish were anesthetized with an ice-cold water solution and 0.05 mL of STZ was administered intraperitoneally (**i.p.**) using an insulin syringe with a 27 gauge needle. The STZ stock solution was prepared by mixing 7 mg of STZ in 1000 µL of sodium citrate buffer (pH-5.5) solution. Thereafter, fish were kept in 1 % W/V glucose solution for 30 minutes to avoid hypoglycemic impact on fish health. After 24 hours, the zebrafish blood drops were collected by gentle application of a 1 mm outer-diameter glass capillary with a needle puller in the caudal vein location as described by Zang *et al.* [33]. The rise in blood glucose level was measured by utilizing a commercial glucometer. The level of blood glucose raised above 15 mM of glucose was considered a diabetic condition. The development of DR progression was raised by intravitreal (**i.vit.**) injection of STZ (20 µL of 7 % w/v of STZ stock solution) on the 7th day in diabetic animals under stereomicroscopic observation.

2.4 Experimental protocol

Five groups of healthy male zebrafish (*Danio rerio*; n = 20) were used in this research.

Group 1: Naive control group. Animals were not involved in the administration of any drugs and chemicals.

Group 2: DR control group: Animals were injected STZ (350 mg/kg; *i.p.* and 20 µL of 7 % w/v of STZ *i.vit.*) as described in the previous section.

Group 3: PBC (50) treatment group. Animals were exposed to PBC (50 mg/L) for 21 continuous days in DR-induced conditions from the 8th day of the study protocol. Dimethyl sulfoxide was used as a solvent for PBC solution preparation by sonication method.

Group 4: PBC (100) treatment group. Animals were exposed to PBC (100 mg/L) for 21 continuous days in DR-induced conditions from day 8 of the study protocol.

Group 5: Dexamethasone (10) treatment group. Animals were exposed to dexamethasone (DEX, 10 mg/L) for 21 continuous days in DR-induced conditions from day 8 of the study protocol. Ethanol was used as a solvent for DEX solution preparation. The summarized procedure of the investigation procedure is shown in Figure 1.

Thereafter, the neurobehavioural monitoring responses *i.e.,* optokinetic motor response (OMR) and startle response (SR) were evaluated on 0, 7, 14, and 21st days as described below (from the 7th day of the first STZ (*i.p.*) injection). On the 22nd day, every animals were used in the collection of blood, retina, and lens tissue for the evaluation of biomarker alterations like plasma homocysteine (HCY); retinal tissue thiobarbituric reactive substances (TBARS) [34]; reduced glutathione (GSH) [35]; tumor necrosis factor-alpha (TNF-α), superoxide dismutase (SOD) and total protein [36] levels. The lens was used for the assessment of polyol pathway markers *i.e.,* sorbitol dehydrogenase (SDH), aldose reductase (AR) activity, and total protein [36] levels as described in the following sections. The details of the study protocol and timelines of assessments are illustrated in the following Figure 2.

2.5 Assessment of zebrafish visual functions

The DR-induced alteration of visual functions *i.e.,* OMR and SR were assessed at variable time interval points *i.e.,* 0, 7, 14, and 21st days. OMR test is working based on stereotyped eye movement in response to the movement of objects in the surrounding environment. SR test response is working based on the generation of reaction to unexpected intense stimuli. The visual startle response is designed to elicit the eye blink reflex and sudden visual-motor response reflex. The information on the evaluation methods is described in the following segment.

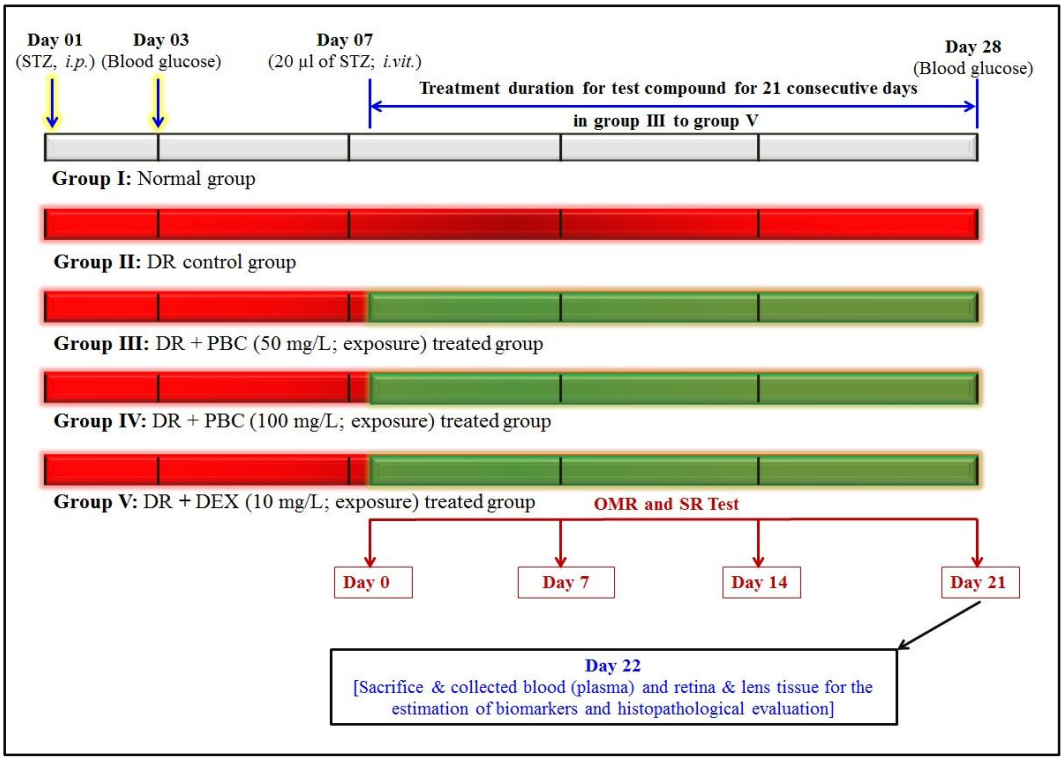

Figure 2: The details of the study protocol and timelines of assessments.

Abbreviations: DEX, dexamethasone; DR, diabetic retinopathy; *i.p.*, intraperitoneal; *i.vit.*, intra-vitreal; OMR, optomotor response; PBC, palm oil mill effluent derived beta carotene; SR, startle response; STZ, streptozotocin.

2.5.1 Evaluation of zebrafish OMR

The OMR test is adopted for the assessment of zebrafish visual acuity functions. It indicates the swimming capabilities against the moving black and white stripes as described by Fleisch and Neuhauss [37] with a slight modification of Fu *et al.* [38]. Briefly, the OMR apparatus has both concentric circular acrylic drums. The inner drum was made up of transparent acrylic sheets whereas the outer drum was made of non-transparent acrylic drum. The inner drum chamber space consisted of 120 mm inner diameter and 100 mm of height. At the center of the inner chamber, a 30 mm diameter and 70 mm height pole was fixed so that fish were able to swim in the space between the center pole and the inner drum wall. The inner drum was with filled water for 30 mm of height. The outer drum consists of 200 mm of width and 100 mm of height and it has 10 mm of width and 80 mm of height vertical slits alternatively. The outer drum was covered with a dark chamber and the inside was fixed with white LED lights. The outer drum was connected to 10 revolutions per minute (RPM) speed (CW, Clockwise and CCW, Counter Clockwise) of a 220V direct current (DC) synchronous motor. It acts as black-and-white strips of movement object stimuli to explore the zebrafish's visual behaviour response in the OMR test as described by Ninkovic and Bally-Cuif [39]. During the OMR assessment, the room light intensity was maintained at 300 lux at the water surface level.

Basel training was given to all the zebrafish one day before the investigation. The zebrafish were put into the

middle chamber with all basic environmental conditions for 3 minutes without rotation of the outer drum. Thereafter, the clockwise revolution of the external chamber was begun to evaluate the fish swimming developments against the visual strips stimuli movement. The Basel preparing preliminary was stopped when the fish could complete five circles. The OMR visual acuity test was performed on 0, 7, 14, and 21st days; by measuring the fish swimming movements against visual stimuli by clockwise and counterclockwise rotation of the outer chamber. The rising number of cycles finished by 4 4-minute periods (2 minutes of clockwise and 2 minutes of counterclockwise rotations of the outer chamber) was noted as spatial frequency threshold (SFT) in the OMR test. The decline in the SFT indicates the loss of visual acuity functions with a correlation of rods and cone cell dysfunctions. Unerringly, zebrafish are more delicate to visual boosts at sunset than at sunrise[26]. Hence, the OMR test was carried out between 3.00 PM to 4.00 PM. The optokinetic motor apparatus for the evaluation of zebrafish visual acuity functions is shown in Figure 3.

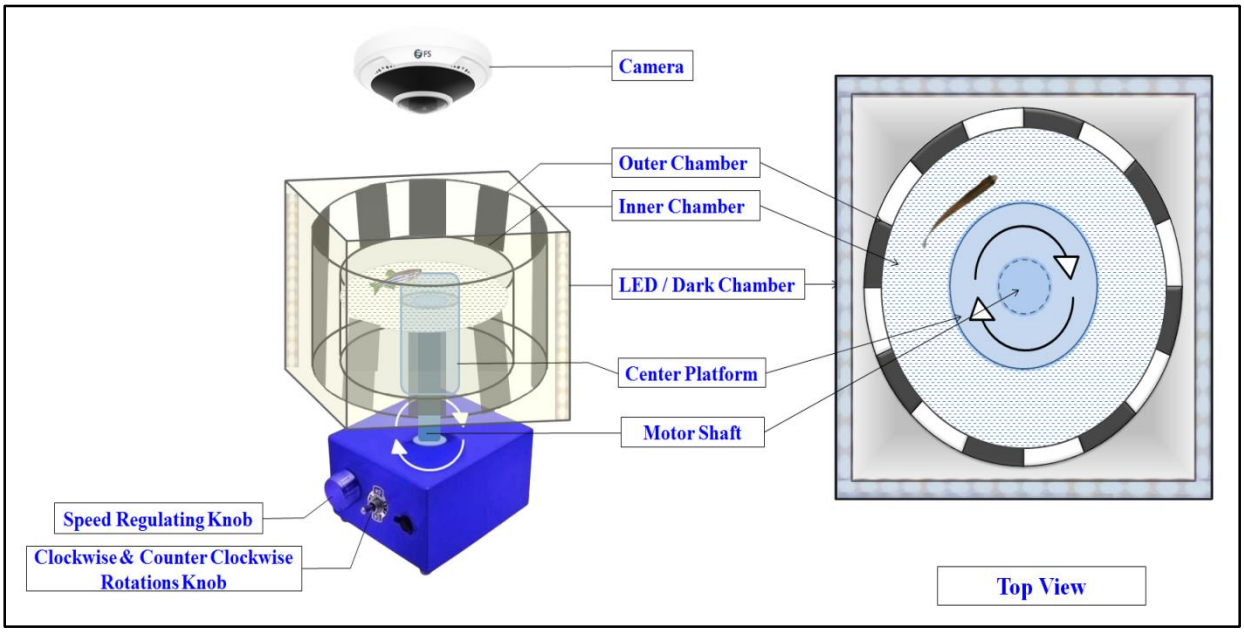

Figure 3: Illustration of OMR response test device for the assessment of zebrafish visual acuity functions.

The above figure helps to understand the design of the optokinetic motor response test apparatus. The response of fish movements was recorded by using a CCTV camera at a top angle and recorded video was used for further analysis of OMR responses.

2.5.2. Assessments of zebrafish startle response (SR)

Zebrafish readily react to unexpected stimuli as startle response (SR). In this condition, animals are showing erratic movements as narrated by Burton *et al.* [40] with a slight change to Wang *et al.* [32]. Shortly, the SR test chamber has a transparent square (25 x 25 cm) chamber with a 10 cm height. The water was topped up to 5 cm. The back side of the chamber (outer surface) was surrounded with waterproof graph paper. The recorder implement was placed at the front side of the SR test apparatus for the noting of animal movements. The animals were allowed to adapt for a 3 3-minute period in

this chamber. Basel swimming behavior was recorded by the count of line passing in 2 2-minute periods. The application of startle stimuli *i.e.*, continuous flashing light (every 10 seconds) by LED strobe light device (300 lux) for 2 minutes was applied. The unusual changes in body position and increase in erratic swimming patterns were noted. The SR recorded the count of line crossings in between 4-minute duration. The SR test was performed in the interval of 4.00 PM to 5.00 PM. The percentage startle response was calculated by using the following formula and the SR test device is illustrated in Figure 4.

$$\%SR = \frac{Number\ of\ line\ crossing\ after\ light\ exposure}{Number\ of\ line\ crossing\ before\ light\ exposure}\ X\ 100$$

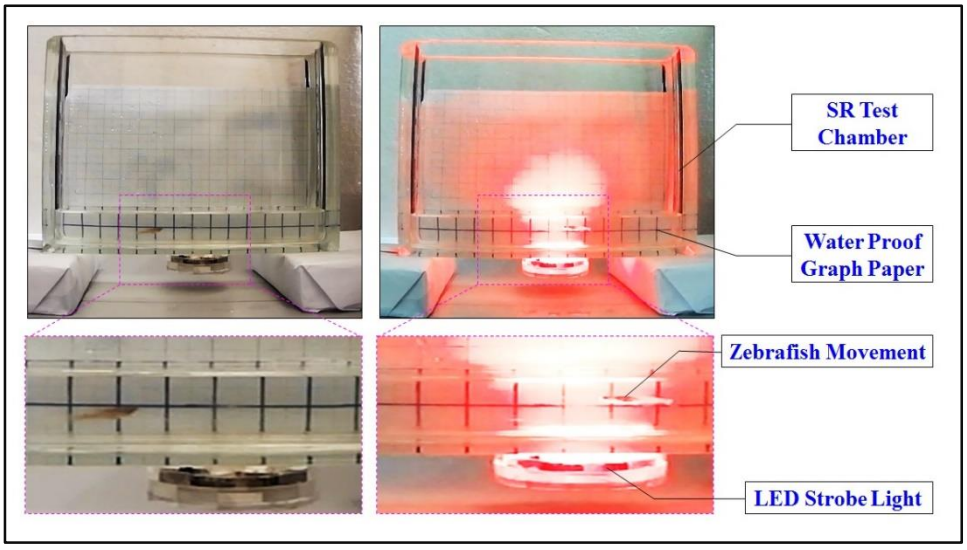

Figure 4: Illustration of SR test device for the assessment of %SR against unexpected flashlight stimuli. *Abbreviations:* SR, startle response; LED, light-emitting diode.

2.6 Estimations of plasma HCY biomarker changes

The blood tests were gathered on the 21st day from zebrafish according to the explanation of Pedroso *et al.* [41] with minor changes from Wang *et al.* [32]. Briefly, the animal was placed in an ice-cold solution (as an anesthetic solution). The diagonal cut was made between anal and caudal fin. Thereafter, the animal was placed in a 2 mL Eppendorf tube which contains 0.1 mL of 11% w/v of sodium citrate solution (as an anti-coagulant). The tubes were vortexed at 112 g centrifugation force for 10 minutes at 4°C. The gathered plasma samples were diluted with sodium citrate solution (11 % w/v; 1:4 ratios) for the estimations of plasma HCY levels.

The plasma HCY levels were estimated by using the commercial HCY ELISA kit (Abbexa Ltd., Cambridgeshire, United Kingdom). Briefly, the 0.1 mL of plasma samples were applied in a microplate reader (ClaIR™, Photon Etc., Montreal, QC, Canada) and mixed with 100 µL of detection reagent A and incubated for 1 hour at 37 °C. Then, the plate was rinsed and discorded with washing buffer. The washing process was done three times. Thereafter, 0.1 mL of detection reagent B was added to the plate and incubated for 1 hour at 37 °C. Then, the washing process was applied five times. Crucially, 90 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogenic substrate was added into each well and incubated at 37 °C. The plate was placed dark chamber to avoid exposure to the light. The reaction was stopped with a mixing of 0.05 mL of stop solution after 20 minutes of incubation period. The standard plot of HCY was prepared with 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 ng/mL of HCY standard. The changes of absorbance were measured immediately at 450 nm wavelength. The HCY content of plasma was calculated by using the following formula.

$$Relative\ Absorbance\ (OD) = OD_{Each\ Well} - OD_{Zero\ Well}$$

The standard plot was made with relative OD value in the X axis as the reference standard and respective HCY concentration of each standard solution in the Y axis. Test sample concentrations were calculated by using the HCY standard plot and multiplied by the dilution factor. The collection of zebrafish plasma sample procedure is illustrated in Figure 5.

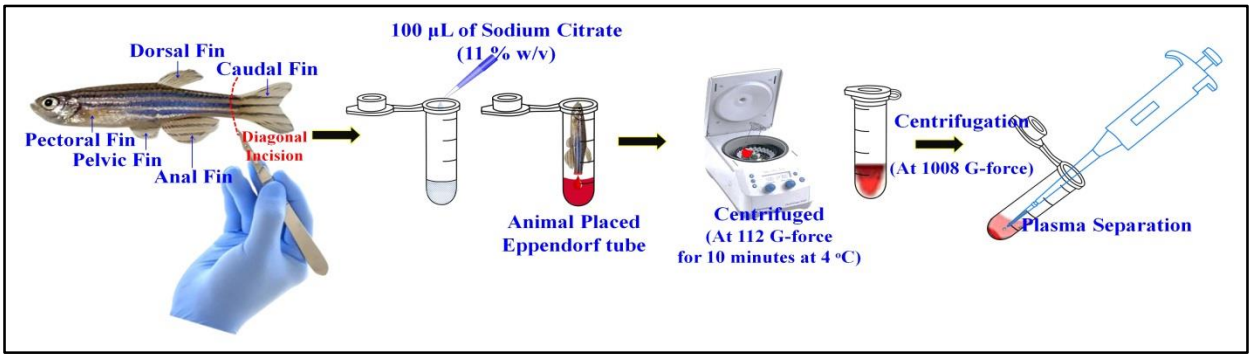

Figure 5: The procedure for the collection of zebrafish plasma samples.

The above illustration reveals the steps involved in the collection of plasma samples for the assessment of biomarker changes.

2.7 Estimations of zebrafish retinal and lens tissue biomarker changes

Zebrafish retina and lens samples were collected and homogenized with a phosphate buffer (pH 7.4) solution. The homogenized samples were vortexed to 1372 g force for 15 minutes at 4 ºC. The retinal tissue samples were used for the quantification of TBARS [34]; GSH, [35]; SOD (Cayman chemical superoxide dismutase assay kit); and TNF-α (ELISA kit method) levels. The aliquots of lens tissue were used for the assessment of SDH (Abcam SDH activity assay kit) and AR (Abcam AR activity assay kit) activity and total protein [36] levels as described in the following sections. The collection of zebrafish retina and lens samples is illustrated in Figure 6.

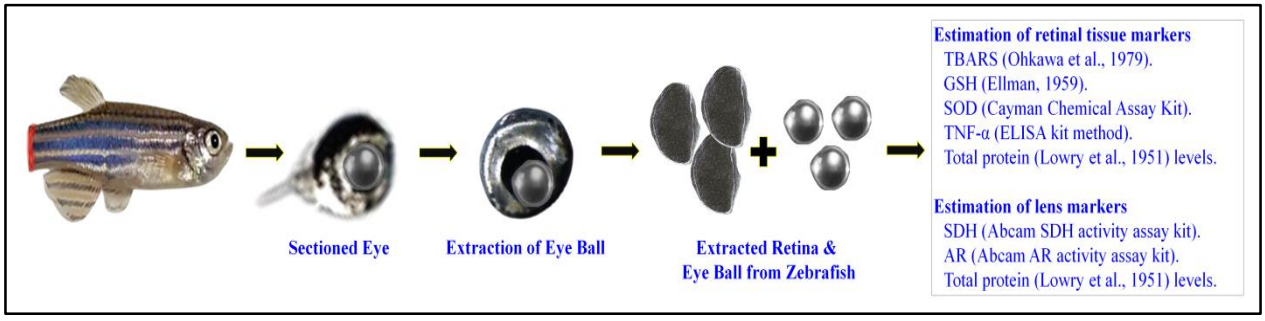

Figure 6: The collection of zebrafish retina and lens samples for the assessment of tissue biomarker changes.

The retina and lens samples help to determine the tissue oxidative stress markers (TBARS, GSH, and SOD); inflammatory biomarker (TNF-α); and polyol pathway markers (SDH and AR) levels. *Abbreviations:* TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha; SDH, sorbitol dehydrogenase; and AR, aldose reductase.

2.7.1 Estimation of retinal TBARS content

TBARS is one of the major markers of oxidative stress. The free radicals make the peroxidation of lipids (cell membrane) leading to the malondialdehyde (MDA) products. The level of tissue TBARS content was quantified as narrated by Ohkawa *et al.* [34]. Briefly, 0.2 mL of retinal tissue aliquot was disparate with 0.2 mL of sodium hydroxide (8.1%) solution; 0.2 mL of sodium dodecyl sulfate (8.1%) solution; 1.5 mL of acetic acid (30%) solution; and 1.5 mL of 2-Thiobarbituric acid (TBA: 0.8%) solution. The final volume was made with 4 mL of tribble distilled water. Thereafter, test tubes were kept at a 90 °C conditioned environment for 1 hour. Thereafter, 100 µL of distilled water was mixed and vortexed at 1792 G-force for 10 minutes. The pink-coloured chromogen absorbance variations were recorded with a spectrophotometer (DU 640B Spectro-photometer, Beckman Coulter Inc., Brea, CA, USA) instruments at 532 nm wavelength. The standard plot was made with 0 - 1000 nano-moles of 1,1,3,3-Tetramethoxypropane per mL solutions. The level of lipid peroxidation was indicated as nmol of MDA molecules per mg of protein.

2.7.2 Estimation of retinal GSH content

GSH is a non-enzymatic endogenous antioxidant molecule and it scavenges the free radicals. Hence, it is also known as a non-enzymatic oxidant marker. The tissue GSH content levels were quantified as narrated by Ellman [35]. Briefly, the aliquot was prepared by homogenate of tissue with 10% w/v of tri-chloroacetic acid (1:1 ratio) solution. The mixtures were vortexed at 4 °C at 112 G-force for 10 minutes. About 500 µL of aliquot was disparated with 2 mL of disodium hydrogen phosphate (0.3 M) solution and 0.25 mL of 0.001 M of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) solutions. Here, DTNB solution was prepared by using sodium citrate (1% w/v) solution. The changes in yellow chromogen absorbance were measured using a spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., Brea, CA, USA) instruments at 412 nm wavelength. The standard plot was made with 0 - 100 micromoles of GSH per mL. The level of retinal tissue GSH was expressed as µmol of GSH per mg of protein.

2.7.3 Estimation of retinal SOD activity

SOD is an enzymatic endogenous antioxidant molecule and it makes the dismutation of superoxide anion to molecular oxygen and hydrogen peroxide. Hence, it is also known as an enzymatic oxidative stress marker. The retinal tissue SOD content levels were estimated as described in superoxide dismutase assay kit (Cayman Chemical, Michigan, United States). Briefly, about 10 µL of aliquot was disparate with 0.2 mL of radical detector and 20 µL of xanthine oxidase. The microplate was maintained at 37 °C for 30 minutes with a shaker. The changes in absorbance were measured using a microplate reader (ClaIR™, Photon ETC., Montreal, QC, Canada) instruments at 450 nm. The A standard plot was prepared with 0 - 200 µL of SOD per mL.

$$SOD\ (U/ml) = \left[\left(\frac{Sample\ Linearized\ SOD\ Rate - Y_{Intercept}}{Slope}\right) x\ \frac{Well\ volume}{Sample\ volume}\right] x\ DF$$

Here, SOD is a superoxide dismutase; and DF is a dilution factor. The level of retinal tissue SOD was indicated as a unit per mg of protein.

2.7.4 Estimation of retinal TNF-α content

The retinal tissue TNF-α content level was estimated by using the commercial TNF-α ELISA kit (Assay Genie, Dublin, Ireland). Briefly, the 100 µL of tissue aliquots were placed in a microplate reader (ClaIR™, Photon Etc., Montreal, QC, Canada) and maintained at 37 °C for 2.5 hours. Then, the microplate was rinsed out with washing buffer. Then, 0.1 mL of biotin antibody was added, and maintained the plate at 37 °C for 1 hour. The second step of washing was to make the microplate wells, followed by mixing the 100 µL of Streptavidin solution & incubating it

with gentle shaking for 45 minutes at 37 °C. Crucially, the microplate washing was made with a washing buffer solution. At the end, 0.1 mL of TMB substrate solution was mixed and the plate was maintained at room temperature for 30 minutes. Thereafter, the stop solution (50 µl) of the reaction was added to each well. The standard plot of TNF-α was prepared with 0, 0.041, 0.102, 0.256, 0.64, 1.6, 4, and 10 ng/mL of TNF-α standard. The variations of absorbance were recorded at 450 nm wavelength. The plasma TNF-α content was calculated by implementing the following formula:

$$Relative\ Absorbance\ (OD) = OD_{Each\ Well} - OD_{Zero\ Well}$$

The standard plot was made with relative OD value in the Y axis as the reference standard and respective TNF-α concentration of each standard solution in the X axis. The concentration of the test sample was calculated from the TNF-α standard curve and multiplied by the dilution factor.

2.7.5 Determination of SDH activity

The lens SDH activity level was estimated as described in the SDH activity assay kit (Abcam Limited., Cambridge, United Kingdom). Briefly, the 0.05 mL of fish lens aliquot was disparated with 50 µL with SDH assay buffer and 50 µL of reaction mixtures. It consists of 50 µL of NADH (1 mM) standard solution; 250 µL of d-Fructose (1.1 M); and 117.5 µL of triethanolamine buffer (100 mM: pH 7.6). The standard plot was made with 0, 2, 4, 6, 8, & 10 nmol of NADH standard. The absorbance changes were noted immediately at 450 nm wavelength; every 20 seconds for 60 minutes at 37 °C. The SDH activity of the fish lens was calculated by using the following formula:

$$SDH\ activity = B\ /\Delta t\ x\ V\ x\ DF$$

Here, B is an NADH content from the standard plot (in nmol); Δt is the reaction time (in minutes); V is a sample volume (in mL); and DF is a dilution factor. The SDH activity level was indicated as mU per mg of protein.

2.7.6 Determination of AR activity

The AR activity level was determined as described in the AR activity assay kit (Abcam Limited., Cambridge, United Kingdom). Briefly, the 50 µL of fish lens aliquots was disparated with 30 µL of phosphate buffer (0.067 M); 60 µL of diluted nicotinamide adenine dinucleotide phosphate (NADPH); 10 µL of DL-glyceraldehyde (5×10^{-4} M). The standard plot was prepared with 0, 20, 40, 60, 80, and 100 nmol of NADPH standard. The variations of absorbance changes were recorded immediately at 340 nm wavelength every 20 seconds for 60 minutes at 37 °C using a UV/Visible spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., California, United States) instrument. The AR activity was calculated by using the following formula:

$$AR\ activity = \frac{B_{Test} - B_{Sample\ Control}}{\Delta t\ x\ M}$$

Here, B is an NADPH amount from the standard curve (in nmol); Δt is a reaction time (in minutes); M is a total protein (in mg), and D is a dilution factor. The AR activity level was indicated as mU/mg protein.

2.7.7 Estimation of total protein levels

The tissue total protein was estimated as narrated by Lowry's method [36]. Briefly, about 10 µL of aliquot was disparated with 10 µL alkaline copper sulfate and conditioned at 37 °C for 10 minutes. Thereafter, 100 µL of Folin's phenol reagent was mixed and conditioned at 37 °C for 10 minutes. The standard plot was prepared with 0, 0.125, 0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 mg of bovine serum albumin (BSA) per mL. The changes in absorbance were measured at 640 nm using a microplate reader (ClaIR™, Photon Etc., Montreal, QC, Canada) instrument. The concentration of tissue total protein was expressed as mg of protein per mL.

2.8 Statistical data analysis

Data were indicated as mean ± S.D. The OMR and SR behavioural test results were analyzed using a two-way analysis of variance (ANOVA) and succeeded by the

Bonferroni post hoc comparison test after the confirmation of the normality test. The biochemical marker changes were examined by one-way ANOVA and succeeded by Tukey's multiple-range tests. This data analysis was made by GraphPad Prism version 5.0 software (Dotmatics, R&D scientific software company, San Diego, United States). A statistically significant was confirmed when the probability (p) value of less than 0.05 ($p < 0.05$).

3. RESULTS

3.1 Effect of PBC in the zebrafish OMR Test

The intravitreal injection of STZ in diabetic animals showed a significant ($p < 0.01$) statistical difference for visual acuity impairment in the OMR test as a sign of reduction of SFT values when differentiated from the normal control group. It indicates that blood-retina barrier dysfunctions were achieved in DR animals by the administration of STZ. The exposure of PBC (50 and 100 mg/L) for 21 continuous days in DR-induced animals showed significant attenuation of the SFT value in a dose-dependent manner (all $p < 0.001$ *vs.* DR group). It is equivalent to the DEX (10 mg/L) treatment group. The result of the OMR test indicates that PBC possesses ameliorative effects on visual acuity functions. The result of the OMR test is depicted in Figure 7.

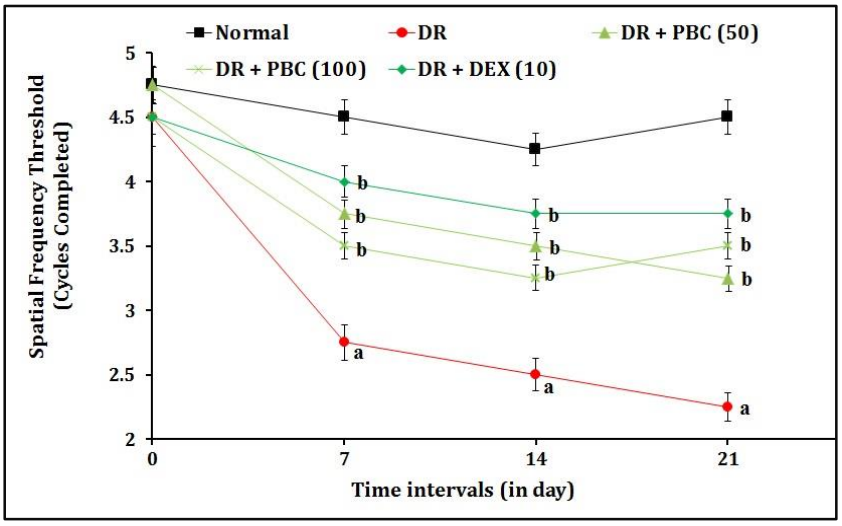

Figure 7: Effect of PBC on STZ-induced DR in OMR test.

The digits in parentheses act for a dose of mg/L. The data were expressed as standard deviation (SD; $n = 20$). $^{a}p < 0.05$ versus the control group; $^{b}p < 0.05$ versus the DR group. *Abbreviations:* DEX, dexamethasone; DR, diabetic retinopathy; PBC, palm oil mill effluent-derived beta-carotene; and SFT, spatial frequency threshold.

3.2 Effect of PBC in the zebrafish SR Test

The intravitreal injection of STZ in diabetic animals showed a significant ($p < 0.01$) statistical difference for erratic movements (as visual impairments) in the SR test as a sign of a reduction of %SR values when differentiated from the normal control group. The exposure of PBC (50 and 100 mg/L) for 21 continuous days in DR-induced animals showed significant attenuation of the %SR value in the SR test in a dose-dependent manner (all $p < 0.001$ *vs.* DR group). It is equivalent to the DEX (10 mg/L) treatment group. The result of the SR test indicates that PBC possesses ameliorative effects on visual impairments. The result of the SR test is depicted in Figure 8.

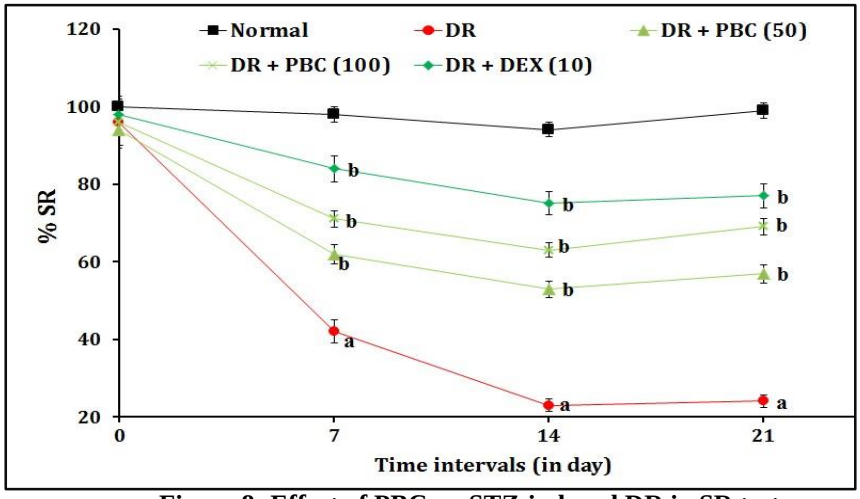

Figure 8: Effect of PBC on STZ-induced DR in SR test.

The digits in parentheses act for a dose of mg/L. The data were expressed as standard deviation (SD; $n = 20$). $^{a}p < 0.05$ versus the control group; $^{b}p < 0.05$ versus the DR group. *Abbreviations:* DEX, dexamethasone; DR, diabetic retinopathy; PBC, palm oil mill effluent-derived beta-carotene; and SR, response.

3.3 Effect of PBC on blood glucose level changes

The intravitreal injection of STZ in diabetic animals showed a significant ($p < 0.01$) statistical difference for the rising of blood glucose levels on days 1, 3, 7, and 28 when differentiated from the normal control group. It indicates that hyperglycemia was achieved and maintained with DR progression by the administration of STZ. The exposure of PBC (50 and 100 mg/L) for 21 continuous days in DR-induced animals showed significant attenuation of the elevated blood glucose value on day 28 in a dose-dependent manner (all $p < 0.001$ *vs.* DR group). It is equivalent to the DEX (10 mg/L) treatment group. The result of blood glucose level indicates that PBC possesses ameliorative potential for diabetic conditions. The result of the blood glucose level changes is expressed in Table 1.

Table 1: Effect of PBC on STZ-induced blood glucose level changes.

Groups	Day 0m (mmol/L)	Day 3 (mmol/L)	Day 8 (mmol/L)	Day 28 (mmol/L)
Normal	5.9 ± 0.4	6.2 ± 0.8	5.8 ± 0.4	6.3 ± 0.6
DR	6.4 ± 0.6	22.5 ± 1.1[a]	24.3 ± 0.7[a]	28.3 ± 1.4[a]
DR + PBC (50)	6.1 ± 0.7	23.7 ± 1.3[a]	22.2 ± 1.1[a]	12.4 ± 1.3[b]
DR + PBC (100)	6.2 ± 0.3	21.4 ± 0.9[a]	25.4 ± 1.0[a]	10.6 ± 0.9[b]
DR + DEX (10)	5.8 ± 0.8	22.3 ± 1.2[a]	23.5 ± 1.3[a]	9.4 ± 1.2[b]

Numbers in parenthesis indicate dose mg/kg. Data were expressed as standard deviation (SD; n = 20). $^{a}p < 0.05$ Vs normal group. $^{b}p < 0.05$ Vs STZ control group. *Abbreviations:* DEX, dexamethasone; DR, diabetic retinopathy; and PBC, palm oil mill effluent-derived beta-carotene

.3.4. Effect of PBC on STZ-induced plasma HCY level changes

The intravitreal injection of STZ in diabetic animals showed a significant ($p < 0.01$) statistical difference for the rising of HCY levels when differentiated from the normal control group. It indicates that plasma HCY contributed to the progression of DR by the administration of STZ. The exposure of PBC (50 and 100 mg/L) for 21 continuous

days in DR-induced animals showed significant attenuation of the above plasma HCY level changes in a dose-dependent manner (all $p < 0.001$ *vs.* DR group). It is equivalent to the DEX (10 mg/L) treatment group. The result of plasma HCY level changes indicates the PBC possesses the ameliorative potential in the regulation of diabetes-associated metabolic vascular toxin (HCY) accumulation. The result of the plasma HCY level changes were expressed in Figure 9.

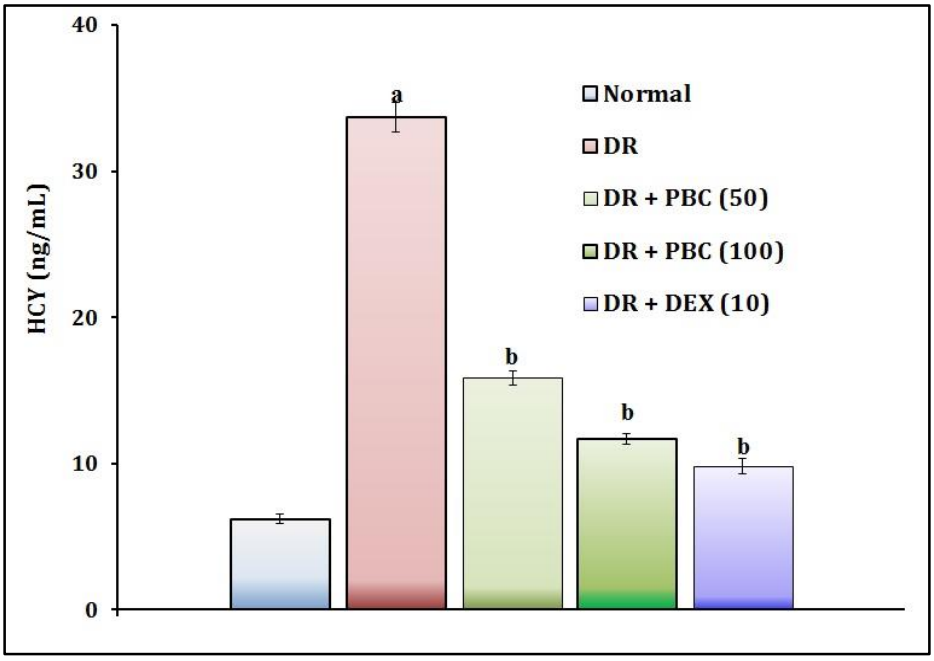

Figure 9: Effect of PBC on STZ-induced plasma HCY level changes.

The digits in parentheses represent a dose of mg/L. The data were expressed as standard deviation (SD; $n = 20$). $^{a}p < 0.05$ versus the control group; $^{b}p < 0.05$ versus the DR group. *Abbreviations:* DEX, dexamethasone; DR, diabetic retinopathy; PBC, palm oil mill effluent-derived beta-carotene; and HCY, homocysteine.

3.5. Effect of PBC on STZ-induced retinal tissue biomarker changes

The intravitreal injection of STZ in diabetic animals showed a significant ($p < 0.01$) statistical difference for the rising of TBARS, TNF-α levels, and reduced levels of GSH and SOD when differentiated from the normal control group. The exposure of PBC (50 and 100 mg/L) for 21 continuous days in DR-induced animals showed significant attenuation of the above changes of retinal tissue biomarkers in a dose-dependent manner (all $p < 0.001$ *vs.* DR group). It is equivalent to the DEX (10 mg/L) treatment group. The result of retinal tissue biomarker changes indicates the PBC possesses ameliorative potential in the regulation of diabetes-associated oxidative stress and inflammatory reactions. The results of the retinal biomarker differences are expressed in Table 2.

Table 2: Effect of PBC on STZ-induced retinal tissue biomarker changes.

Groups	TBARS (nmol/mg of Protein)	GSH (µmol/mg of Protein)	SOD (U/mg of Protein)	TNF-α (ng/mg of Protein)
Normal	1.52 ± 0.02	22.32 ± 1.1	54.21 ± 1.1	3.17 ± 0.03
DR	3.76 ± 0.06[a]	6.87 ± 1.3[a]	7.93 ± 1.6[a]	48.82 ± 0.09[a]
DR + PBC (50)	2.27 ± 0.07[b]	14.94 ± 1.5[b]	28.76 ± 0.9[b]	13.59 ± 0.08[b]
DR + PBC (100)	2.04 ± 0.03[b]	11.85 ± 0.9[b]	20.38 ± 1.2[b]	9.63 ± 0.12[b]
DR + DEX (10)	1.71 ± 0.05[b]	8.27 ± 1.2[b]	13.71 ± 1.4[b]	5.73 ± 0.08[b]

Digits in parentheses indicate a dose of mg/L. Data were expressed as standard deviation (SD; n = 20). [a]$p < 0.05$ Vs normal group. [b]$p < 0.05$ Vs DR group. *Abbreviations:* DEX, dexamethasone; DR, diabetic retinopathy; GSH, reduced glutathione; PBC, palm oil mill effluent-derived beta-carotene; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; and TNF-α stands for tumor necrosis factor-alpha.

3.6. Effect of PBC on STZ-induced lens tissue biomarker changes

The intravitreal injection of STZ in diabetic animals showed a significant ($p < 0.01$) statistical difference for the rising of SDH and AR levels when differentiated from the normal control group. It indicates that STZ alters the polyol pathway with the progression of DR. The exposure of PBC (50 and 100 mg/L) for 21 continuous days in DR-induced animals showed significant attenuation of the above variation of biomarkers in a dose-dependent manner (all $p < 0.001$ *vs.* DR group). It is equivalent to the DEX (10 mg/L) treatment group. The result of lens tissue biomarker variations indicates the PBC possesses ameliorative potential in the regulation of diabetes-associated polyol pathways alteration. The result of the lens tissue biomarker changes were expressed in Figure 10.

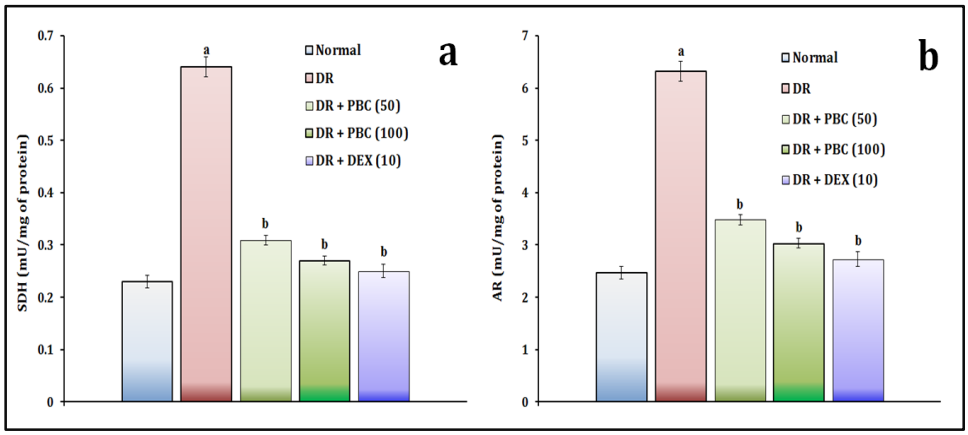

Figure 10: Effect of PBC on STZ-induced lens tissue biomarker changes.

Panel A indicates the SDH activity and panel B indicates the AR activity. The digits in parentheses represent a dose of mg/L. The data were expressed as standard deviation (SD; $n = 20$). [a]$p < 0.05$ versus the control group; [b]$p < 0.05$ versus the DR group. *Abbreviations:* AR, aldose reductase activity; DEX, dexamethasone; DR, diabetic retinopathy; PBC, palm oil mill effluent-derived beta-carotene; and SDH, sorbitol dehydrogenase activity.

4. DISCUSSION

The intravitreal injection of STZ in diabetic animals showed statistically significant ($p < 0.05$) changes in DR-associated visual-motor reflex changes via blood-retina barrier dysfunctions which are assessed by OMR and SR tests. Furthermore, it also alters the plasma (glucose and HCY) levels), and tissue biomarkers (TBARS, GSH, TNF-α, SOD, SDH, and AR activities) which indicate the amelioration of DR-associated oxidative stress, inflammatory reactions, and alteration of polyol pathways. However, the exposure of PBC (50 and 100 mg/L) and DEX (10 mg/L) for 21 continuous days showed significant betterment of STZ-induced DR and their blood-retina barrier dysfunctions and changes in biomarkers. STZ is known to induce diabetes mellitus along with the induction of local inflammatory reactions via the production of reactive methylcarbonium ions which alkylate the cellular DNA content and lead to cell damage [13,42]. Experimentally, the *i.vit.* administration of STZ in zebrafish is known to accelerate the DR progression and alter the visual acuity functions [32]. The interchangeable results were noted in this investigation *i.e.*, decreasing the SFT value in the OMR test and reducing the percentage startle response value in the SR test in 7, 14, and 21st days with the administration of PBC (50 and 100 mg/L) and DEX (10 mg/L) treatments.

In diabetic conditions, circulatory glucose readily reacts with metabolic pathways leading to accumulating the AGE products which leads to enhanced oxidative stress in retinal tissue [43]. The retinal tissue is highly sensitive to free radicals associated with oxidative damage and it plays a pivotal part in the subsequent changes of inflammatory reactions via generation of inflammatory cytokines (mainly TNF-α). The combined action of oxidative stress and inflammatory cytokines enhances the acceleration of cellular DNA damage and the development of DR [44]. A similar effect was observed in the present study, the exposure of PBC and DEX attenuated the STZ-associated TBARS, GSH, TNF-α, and SOD levels. DEX is a steroidal (glucocorticoid) medication and it is widely used for the treatment of various inflammatory disorders including DR via the reduction of hyperglycemic & oxidative stress; and inflammatory reactions [45]. It is a multi-targeted medicine, similarly, natural medicine also possesses the multi-targeted action without harmful actions to the body [46]. The abundant free radicals also interact with the cellular lipid membrane and make the lipid peroxidation which leads to cell damage [47]. Furthermore, free radical generation in diabetic animals is known to alter the enzymatic oxidative defensive system in the retina [48]. Similarly, our study also found that STZ-induced DR was shown to reduce the SOD activity levels in retinal tissue.

DR is one of the metabolic complications in diabetes mellitus which is mainly due to the alteration of polyol pathways [49]. The markers of polyol pathways are SDH and AR activities. The rising of the SDH and AR activities indicates the worsening of retinal tissue due to the activation of polyol pathways [50,51]. The changes in polyol pathways in DR are observed in lens tissue [52]. The current results revealed that STZ induces the DR complication via the rising of the SDH and AR activities. However, the multi-targeted natural medicine *i.e.*, PBC attenuates the DR via regulation of polyol pathways. PBC possesses glucose regulatory actions via the regulation of oxidative stress and inflammatory reactions [13]. Besides, our previous publication shows that BC possesses the ameliorative potential of DR via regulation of oxidative stress markers i.e., reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARSs), and catalase activity in diabetic mice [27]. Further, BC also possesses an ameliorative effect against the α-amanitin-induced nephrotoxicity via polyol pathways in rat kidneys [53]. Moreover, BC attenuates eye diseases like cataracts, age-related macular degeneration, and DR via free radical scavenging, anti-apoptosis, and regulation of mitochondrial function and inflammation [54]. The polyol pathway mechanism is reported in the progression of DR

[55]. However, there is no direct connection was reported between the BC action in the progression of DR via polyol pathway in experimental animal models (rodents & zebrafish). This is the first research report that shows evidence that BC produces the ameliorative action of DR in the zebrafish model via the polyol pathway mechanism.

Carotenoids consist of 8-isoprene units $(C5H8)_8$ skeleton (tetraterpene pigments) and it is a natural lipophilic compound present in plants (*i.e.,* (corn, carrots, oranges, and tomatoes); and microorganisms (*i.e.,* fungi, algae, and bacteria). Carotenoid compounds are known to possess potential therapeutic actions in DR [56]. The molecular mechanism of PBC is known to inhibit the TNF-α associated induced epithelial cell injury [57]. Furthermore, beta-carotene is also known to inhibit the SOD via regulation of mitogen-activated protein kinases (MAPK), nuclear factor–kappa B (NF-κB), and nuclear factor erythroid 2-related factor 2 (Nrf2) signaling [58]. However, the exact action of PBC action in the regulation of polyol pathways has not been reported yet [59][59][58][58][57][57][57][59]. Hence it is the first report that, PBC possesses the regulatory actions on polyol pathways. Moreover, PBC also converts to retinal (vitamin A) in the retina with the help of β-carotene cleavage enzyme *i.e.,* β-carotene oxygenase 1. Hence, the pro-vitamin A or vitamin A precursor *i.e.,* beta-carotene attenuates the diabetes mellitus-associated visual impairments. Hence, it can be utilized for the treatment of DR because of its multi-targeted therapeutic response. However, substantial research is expected to investigate the involvement of polyol and other pathways in the regulation of DR pathological conditions in higher vertebrate animal models before being in clinical trials. The summary of PBC molecular action in the amelioration of STZ-induced DR in zebrafish animal model has been illustrated in Figure 11.

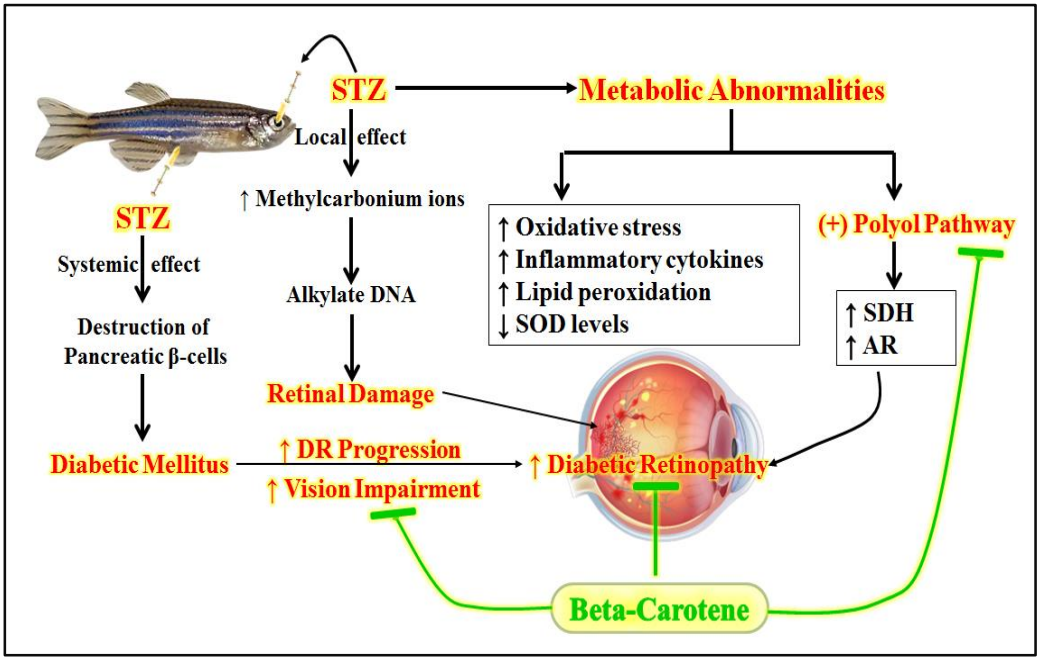

Figure 11: Molecular actions of PBC in the amelioration of STZ-induced DR in zebrafish animal model.

Abbreviations: AR, aldose reductase; DNA, deoxyribonucleic acid; DR, diabetic retinopathy; SDH, sorbitol dehydrogenase; SOD, superoxide dismutase; and STZ, streptozotocin.

CONCLUSIONS

The current study results revealed that the exploration of PBC attenuates the DR-associated visual acuity responses in the OMR and SR tests together with changes in biomarkers *i.e.*, the elevation of GSH & SOD activity levels; decrease of plasma glucose & HCY; and tissue TBARS, TNF-α, SDH & AR levels. Hence, PBC can be used for the treatment of diabetes mellitus-associated microvascular obstructions and dysfunctions because of its anti-oxidative, and anti-inflammatory actions; and regulation of metabolic toxin & polyol pathways. Nevertheless, this study will reach out to investigate retinal–barrier functions with connection to endothelial growth factor and neutrophil recruitment pathways for the effective therapeutic management of DR progression.

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Conflict of interest

The authors declare no conflict of interest.

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سلائف فيتامين أ: بيتا كاروتين يخفف من اعتلال الشبكية السكري الناجم عن الستربتوزوتوسين لدى ذكور الزرد البالغين عن طريق تنظيم مسار البوليول

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ملخص

اعتلال شبكية العين بسبب مرض السكر يعرف على أنه اضطراب وعائي عصبي تقدمي بسبب تلف الأوعية الدموية في شبكية العين. يعمل البيتا كاروتين ككروموفور في شبكية العين ويبدأ في نقل الصور وصيانة خلايا الظهارة. BC موجود في النفايات السائلة من مطحنة زيت النخيل والتي تسمى .PBCتهدف الدراسة الحالية إلى البحث عن دور PBC في الحماية من DR في نموذج حيواني وهو الزرد. تم استخدام المجموعات الخمس من دانيو ريريو الصحية في هذه الدراسة. تم تحقيق اعتلال الشبكية السكري عن طريق إعطاء الستربتوزوتوسين (STZ) داخل الصفاق متبوعًا بإعطاء STZ داخل الصفاق في اليوم السابع. تعرضت الحيوانات إلى جرعتين من 50) PBC و 100 مجم / لتر) بالإضافة إلى الديكساميثازون (DEX) لمدة 21 يومًا متواصلة. تم تقييم السلوكيات البصرية المرتبطة بـ DR، مثل الاستجابة الحركية البصرية (OMR) والاستجابة المفاجئة (SR) جنبًا إلى جنب مع التغيرات الكيميائية الحيوية، مثل الجلوكوز في البلازما والهموسيستين ((HCY وبيروكسيد الدهون، وانخفاض الجلوتاثيون (GSH)، وعامل نخر الورم ألفا ((TNF-α، ديسموتاز الفائق أكسيد (SOD)ومستويات البروتين الكلي. تم استخدام العدسات لتقييم مؤشرات مسار البوليول، مثل نشاط هيدروجيناز السوربيتول ((SDH) ونشاط إنزيم الألدوز المختزل (.(ARأظهرت النتائج أن PBC تخفف من مشاكل اعتلال شبكية العين الناجمة عن مرض السكر DR من خلال تنظيم تشوهات المسار الكيميائي الحيوي بطريقة مشابهة للمجموعة المعالجة بـ DEX. ومن ثم، يمكن استخدام PBC لإدارة وتخفيف اثار DR بسبب دوره فى الإجراءات التنظيمية لمسارات مكافحة ارتفاع السكر في الدم، ومضادات الأكسدة، والمضادة للالتهابات، والبوليول.

الكلمات الدالة: اختزال ألدوس، الجذور الحرة، الحقن داخل الجسم الزجاجي، استجابة المحرك البصري، النفايات السائلة مطحنة زيت النخيل، سوربيتول ديهيدروجينيز.

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Therapeutic Effect of Propolis against Biofilm Gene Expression in *Candida albicans*

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ABSTRACT

The sticky substance called propolis is made from plants and is produced by honeybees. It has been used as a folk remedy since ancient times, and it has numerous pharmaceutical benefits, such as antibacterial and antifungal. The objective of this work was to determine the impact of propolis on the expression of three genes (*Ece1*, *Sap5*, and *Als3*) known to be implicated in the development of *C.albicans* biofilms and define the minimum inhibitory concentration of propolis required for this purpose. The XTT test was used to assess the anti-biofilm activity of propolis in order to ascertain the formation of biofilm on 100 *C. albicans* isolates from stool samples and calculate the minimum inhibitory concentration of propolis that inhibits the biofilm of *C. albicans* during 24 and 48 h. Finally, the impact of propolis on the expression of the *Ece1*, *Sap5*, and *Als3* genes in *C. albicans* was examined using a real-time polymerase chain reaction and compared with the results that appeared in the gene expression of the biofilm *C. albicans* untreated propolis during 24 and 48 h, and it was considered a control. Through comparison, biofilm formation was found to decrease as propolis concentration and time increased. Accordingly, the MIC of propolis was 40% w/v, and its minimum fungicidal concentration (MFC) was 50% (w/v) in biofilm-forming *C .albicans*. Additionally, gene expression level analysis revealed a decrease in *Ece1*, *Sap5*, and *Als3* expression levels with propolis treatment during 24 and 48h.

Keywords: Expression; Ece1; Sap5; Als3; genes.

INTRODUCTION

A healthy human body with a functioning immune system naturally contains *C. albicans* in its microbiome (1). However, several factors combine to create a balance disorder that facilitates the fast growth of this fungus and infection (2, 3). *C. albicans* infection can be caused by a number of factors, including when the immune system is suppressed or there is a disturbance in the host environment. *C. albicans* can rapidly transition to pathogens that can cause various infections. For example, it is well recognized that *C. albicans*' virulence factors, particularly its involvement in biofilm formation, account for the fungus' resistance to both the immune and antifungal systems (4). *C. albicans* biofilm formation starts in the lab when round yeast cells stick to a solid surface. The subsequent phase of biofilm formation involves cells' growth and the attached cells' initial filamentation. The next step is biofilm maturation, which produces a complex network of multiple layers of polymorphic cells encased in an extracellular matrix. These cells include round yeast cells, pseudohyphal cells, ellipsoidal cells joined end to end, hyphal cells, and chains of cylindrical cells. The extracellular matrix gives the biofilm a thick, structured appearance and protects it from physical and chemical damage (5, 6). Mature biofilms usually form in less than 24 hours, and when viewed under a microscope, they appear as an ordered collection of various cell types. To the unaided eye, they appear as a cloudy surface structure on top of a solid surface. The least researched stage of *C. albicans* biofilm development is the dispersal stage,

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which is the last stage of biofilm development, where some round yeast cells scatter from the biofilm to seed new sites (7, 8).

Propolis is one of the natural product's alternative therapies. Honeybees naturally produce a substance called propolis (9). Brazilian propolis breaks the extracellular polymeric substance down, which also causes cell death and prevents *C. albicans* from forming biofilms in vulvovaginal candidiasis (10). A flavonoid is one of the substances in propolis that has been shown to have antibiofilm properties, such as pinocembrin, apigenin, chrysin, and kaempferol (11). However, the kind of plant the bees live in, when the propolis is collected, and where the propolis is from can all affect its composition (12).

C. albicans possesses a particular class of proteins called adhesins, which help the organism adhere to host cells, other microbes, abiotic surfaces, and other *C. albicans* cells. There are eight members of the *C. albicans* adhesions family, which are agglutinin-like sequence (Als) proteins (Als1–7 and Als9) (13). *Candida* species produce two main families of histolytic enzymes: phospholipases (PL) and secretory aspartyl proteinases (Sap) (14). Among the virulence factors in *C. albicans* that have been studied the most are the secreted aspartic proteases (Saps) (15).

Numerous biofilm genes code for predicted or known cell wall proteins. These proteins are particularly interesting because heterologous expression studies suggest that *Als1* and *Als3* have roles in cell-substrate or cell-cell adhesion (16). Previous research has shown that the development of biofilms on mucosal surfaces is linked to the expression of genes from the *Als* and *Sap* gene families, and the hyphae-specific protein *Ece1*(17). *C.albicans*' ability to form hyphae is a crucial component of its virulence because it allows the fungus to adhere to host cells, invade them, and eventually spread throughout deep tissues (18).

The purpose of this study was to ascertain the effect of propolis on the expression of three genes (*Ece1*, *Sap5*, and *Als3*) that are involved in *C. albicans* biofilm formation. Also, this experiment was to find out whether propolis could prevent the growth of *Candida* biofilms and the minimum concentration of propolis needed to accomplish this.

MATERIALS AND METHODS

Sample collection

A total of 100 stool samples were examined for isolated *C. albicans*. Samples were taken from gastrointestinal patients, ranging in age from 1 to 50 years, who visited the pediatric teaching hospital in Samawa City from October 2021 to May 2023. The Al-Muthanna University ethics committee gave its approval for this study.

Candida albicans isolation

Sterile phosphate-buffered saline (PBS) was used to dilute small samples of freshly provided stool specimens, which were then thoroughly vortexed in a 1.5 ml Eppendorf tube. About 100 microliters of this suspension were distributed across a minimum of three solid culture media, which were always Potato Dextrose Agar (PDA), Yeast Peptone Dextrose Agar supplemented with 0·05 g/L chloramphenicol, and Sabouraud Dextrose Agar with 0·05 g/L chloramphenicol. The plates were then incubated for up to 60 hours at 37°C in an aerobic incubator. Once microbial growth was detected, each plate was sealed and kept at 4°C until it was time for additional processing.

Serum is added to promote the growth of *C. albicans*, and when the yeast cells are examined under a light microscope, they resemble large cocci and develop into germ tubes. When *C. albicans* was isolated and identified using Sabouraud dextrose agar and CHROM agar, the colony of the fungus looked green. API (Analytical Profile Index) Candida (HIMDIA/India) employed the biochemical test for *C. albicans* detection to validate the identification of the morphological, cultural, and biochemical characteristics required to differentiate between *Candida* species and to demonstrate that *Candida* can utilize different types of sugar.

Propolis collection

Propolis was taken directly from honeybee hives in Samawah city from 21 March to 21 June 2022. These hives

reliant on apricot, apple, and grape trees for their sustenance. The models were kept in 200 ml sterile, tightly sealed bottles. Each bottle was then labeled with specific information, such as the model type and the collection date until use (19).

Minimum Inhibitory Concentration (MIC) determination

The macro broth dilution assay was used to calculate the MICs of natural propolis against the biofilm of *C. albicans*. Propolis was diluted in YPD (Yeast extract Peptone Dextrose) broth in steps of 80–5% w/v. Under vigorous shaking (120 revolutions per minute), The cultures were incubated at 35 °C for 36 hours. Using the broth from the incubation period, 10^3 CFU (Colony Forming Unit) of *Candida* were aseptically injected into Petri dishes that contained Sabouraud dextrose agar. Following a 48-hour period, the colonies' growth was evaluated, and the MIC of propolis (w/v) was found to be the one that prevented *C. albicans* from growing significantly (20).

Formation of Biofilms

The XTT(2,3-bis(2-metoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt) reduction assay was used to assess the biofilms' growth in 96-well flat-bottomed plates. Utilizing a technique derived from Lal *et al*. (21) and polystyrene microtiter plates. The kinetics of biofilm inhibition were studied using various MIC dilutions of propolis in YPD broth (5% w/v, 10% w/v, 20% w/v, 40% w/v, and 80% w/v) in order to ascertain whether propolis could prevent the formation of *Candida* biofilms and to find the minimum concentration of propolis that could do so. Every MIC dilution in a microtiter plate was analyzed in at least 7 wells. The microtiter plate wells were filled with aliquots of each dilution, each holding 190 milliliters. 10 milliliters of YPD broth containing 5×10^8 CFU/ml were used to cultivate *C. albicans* for 48 hours. To ensure uniform distribution and adherence to the well surfaces, 10 microliters of this 48-hour culture were added to each well and incubated for 1.5 hours at 37°C in an orbital shaker operating at 75 rpm. In order to remove non-adherent cells, adherent cells were carefully washed twice in sterile phosphate buffered saline (PBS) with a pH of 7.4, being cautious not to disturb the adherent cells. This process was done after 1.5 hours. Following washing, 200 ml of a second aliquot of the same propolis dilution in sterile YPD broth was added to each plate well. After that, the plates were kept in the same incubator for 48 hours so that the biofilms could settle in and grow. As a control, 200 ml of autoclaved YPD broth was added to each of the seven micro-titer plate wells. The broth could be positive (containing *Candida*) or negative (not containing *Candida*). The plate was then incubated at 37°C for 48 hours (21).

The purpose of the experiment was to determine whether propolis could disrupt established *C. albicans* biofilms. Ten milliliters of 5 x 108 CFU/ml were used to cultivate biofilms in 96-well microtiter plates. Place C. albicans in YPD onto the microtiter plate. To ensure even dispersion and adherence to the well surfaces, the plate was put in an orbital shaker set to run at 75 rpm for 1.5 hours at 37° C. After an hour and a half, non-adherent cells were extracted by gently washing them twice in sterile PBS (phosphate buffered saline), which had a pH of 7.4. To promote appropriate adhesion and the formation of biofilms in the absence of propolis, 150 milliliters of sterilized YPD broth was added to each well. The plate was then incubated again for 24 to 48 hours at 37 degrees Celsius. 200 ml final volumes of various propolis concentrations (5% w/v, 10% w/v, 20% w/v, 40% w/v, and 80% w/v) were added to each well in the YPD broth. After that, the plate was stored for 48 hours at 37°C. The XTT reduction assay was used for quantification in each triplicate experiment (22).

Biofilm Evaluation with the XTT Reduction Assay

The cells in the biofilms were measured using the XTT assay following propolis treatment. Used a filter with a pore size of 0.22 mm to filter and sterilize after making the XTT solution (1 mg/ml in PBS). Prior to the commencement of every assay, the menadione solution and XTT solution were

mixed at a 5:1 (v/v). To stop the adherent biofilms from growing further, 2.5% glutaraldehyde was applied for five minutes to the microtiter plate wells after three PBS cleanings. Following the removal of the fixative, two PBS washes were applied to the wells. In all but the control well, which had no biofilm, 1 mL of PBS was added to the XTT-menadione solution. After that, the MTPs (Metyrapone) were maintained in the dark for two hours at 37 °C. After the incubation period, 75 ml of XTT-menadione solution was added to each well on a fresh microtiter plate, and spectrophotometry was used to measure the absorbance at 492 nm (22).

RNA isolation and RT-PCR

The PrestoTM Mini gDNA Yeast Kit was used to extract total RNA from *C. albicans* culture cells and co-culture them with propolis in accordance with the manufacturer's instructions. The concentration and degree of RNA presence were determined using a Nanodrop-1000 UV-VIS spectrophotometer. For the agarose gel electrophoresis, RNA templates were employed. The cDNA Synthesis SuperMix Kit and EasyScript One-Step Gdna Removal were used to perform the reverse transcription PCR cDNA synthesis. One cycle at 42°C for 30 minutes and 85°C for 5 seconds was the RT-PCR setup (23).

mRNA transcripts were detected using real-time PCR with the Mx3000p (Agilent Technologies, USA). 10 mM primers were used to prepare the PCR components, which were then amplified by the genes in **Table 1** (24). In this experiment, the housekeeping gene beta-actin is amplified in the forward direction. 5'-CGTCGGTAGACCAAGACACC-3' and the opposite direction 5'-CCCAGTTGGAGACAATACCGT-3', A final volume of 20 µl was achieved by adding nuclease-free water, 200 ng of cDNA, and 10 µl of Luna universal qPCR master mix. The following parameters were used to run the PCR: thirty seconds at 94°C, 45 cycles of 94°C for five seconds, and thirty seconds at 60°C. Then, a dissociation curve was employed, with a single cycle lasting 1.0 minute at 95°C, 30 seconds at 55°C, and 30 seconds at 95°C (24).

Table (1): The PCR Primers for Real-Time Reverse Transcriptase

Genes	primer	Product size
Sap5	Forward 5'-CCAGCATCTTCCCGCACTT- 3' Reverse 5'-GCGTAAGAACCGTCACCATATTTAA- 3'	71bp
EceI	Forward 5'-CCAGAAATTGTTGCTCGTGTTG-3' Reverse 5'-CAGGACGCCATCAAAAACG-3'	138bp
Als3	Forward 5'-CAACTTGGGTTATTGAAACAAAAACA-3' Reverese 5'-AGAAACAGAAACCCAAGAACAACCT-3'	80bp

Statistical Analysis

Different means of biofilm biomass (absorbance) were compared using the ANOVA and t test. Data analysis was performed using GraphPad software.

RESULTS

MIC determination

Propolis exhibited concentration-dependent inhibition of *C. albicans* growth. Propolis's MIC was 40% (w/v), and its minimum fungicidal concentration (MFC) was 50% (w/v) in biofilm-forming *C .albicans*. The MFC required to eliminate 99.9% of the inoculum. In most cases, the MFC value exceeds the MIC value. Growth curves for yeast exposed to 40% (w/v) propolis for 24 hours revealed a reduction in both the total number of cells and the growth rate when compared to cell growth without propolis (Figures 1 and 2). There was no evidence of *C. albicans* growth in the growth assays using 50% (w/v) propolis. Biofilms were allowed to establish for a full day before being incubated for varying periods of time with and without 40% w/v propolis. The biofilm biomass was then measured in order to track the efficacy of propolis over

time. During this study, a decrease in the biofilm biomass values of *C. albicans* was observed when propolis was added to it, in contrast to the values when propolis was not added and was considered a control, especially at 24 to 48 hours.The results of the one-way ANOVA test indicated a significant difference between the treatment group and the control group at p< 0.01.

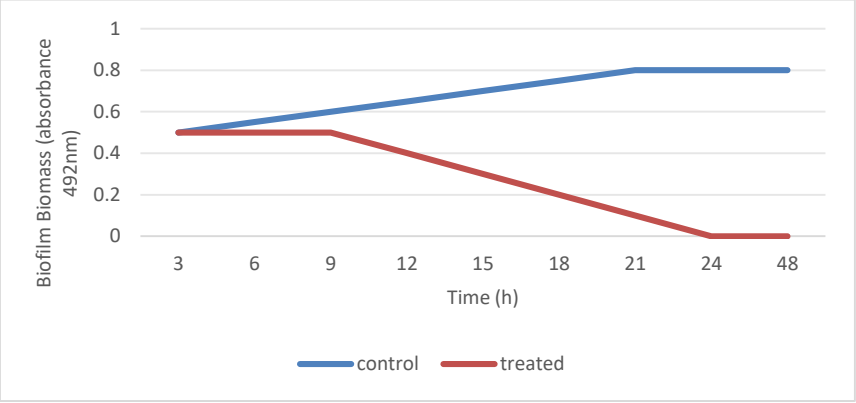

Figure (1): Growth evaluations of propolis-treated established *Candida albicans* biofilms during 24 to 48 h. (F=25.71291, P< 0.01)

Reducing the Formation of Biofilms

It achieved this by using propolis in YPD broth at varying concentrations (5% w/v, 10% w/v, 20% w/v, 40% w/v, and 80% w/v). It was found that the concentration of propolis affected the inhibition of biofilm formation. propolis concentrations less than 10% w/v were shown to neither inhibit nor promote the growth of biofilm (Figure 2). Biofilm formation was considerably inhibited by concentrations greater than 10% w/v. The control and treatment groups differed significantly at p< 0.01, according to the one-way ANOVA test analysis.

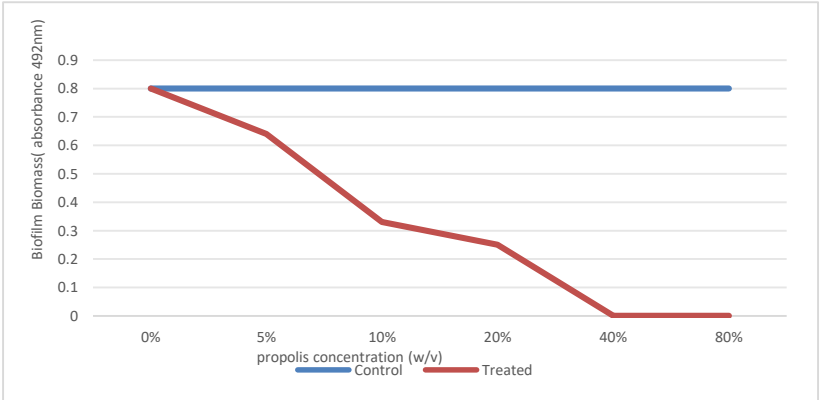

Figure (2): Propolis's impact on the development of *C. albican* biofilms. Propolis's MIC was 40% (w/v), and its minimum fungicidal concentration (MFC) was 50% (w/v) in biofilm-forming *C .albicans* during 24 to 48h. (F=11.90597, P< 0.01)

Transcriptase real-time PCR

Reverse transcription real-time PCR was used to measure the relative expression of the implicated genes (*Ece1*, *Sap5*, and *Als3*) in *C. albicans* throughout the biofilm experiment for 24 and 48 hours.

The expression of the biofilm genes developed by *Candida albicans* was evaluated by quantitative reverse transcriptase PCR in this study. Biofilm genes (*Sap5*, *EceI*, and *Als3*) increased dramatically in expression over time without the treatment of propolis, and It is considered a measure of control when compared with the genes encoding the biofilm treated with propolis (Figure 3). According to the t-test analysis, the biofilm genes expression was a $p>0.05$, considered statistically nonsignificant.

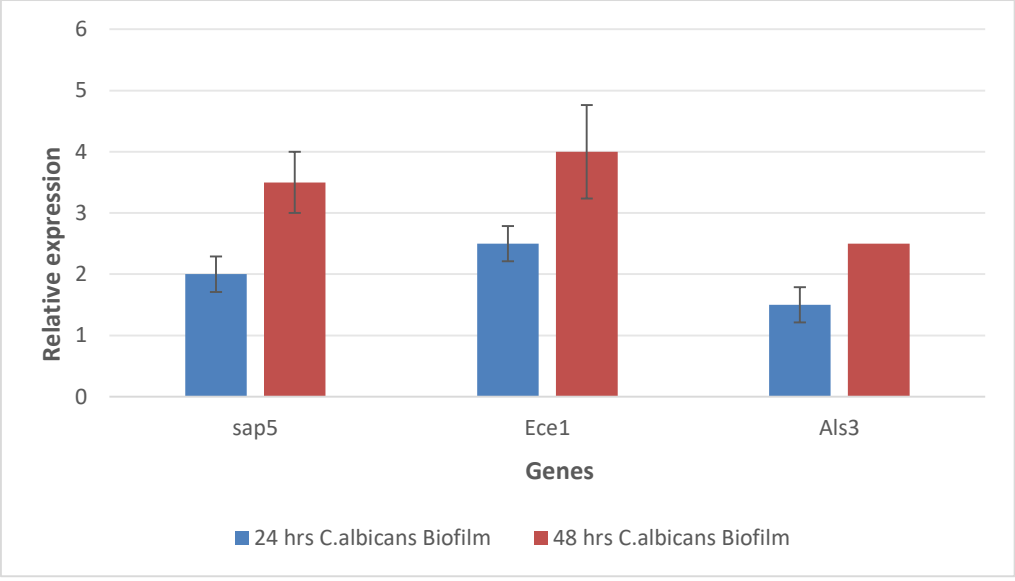

Figure (3): the relative expression of the implicated genes (*Ece1, Sap5, and Als3*) in *C. albicans* during the biofilm experiment without treated propolis and it is considered as control for 24 and 48 hours using reverse transcription real-time PCR. (t= 2.5298, df=4, standard error of difference = 0.527) p>0.05 was considered statistically nonsignificant

C. albicans cells exposed to propolis had less biofilm formation for 24 hours, according to results from a real-time PCR using the specific primers *Ece1*, *Sap5*, and *Als3*. Figure 4 shows that the *Als3* (0.5), *Sap5* (1), and *Ece1* (1.5) genes were treated with propolis, respectively. This indicates the inhibitory role of propolis on the *C. albicans* biofilm within 24 hours compared to the control gene expression. The expression of the biofilm genes, according to the t-test analysis, was a $p>0.05$, considered statistically nonsignificant.

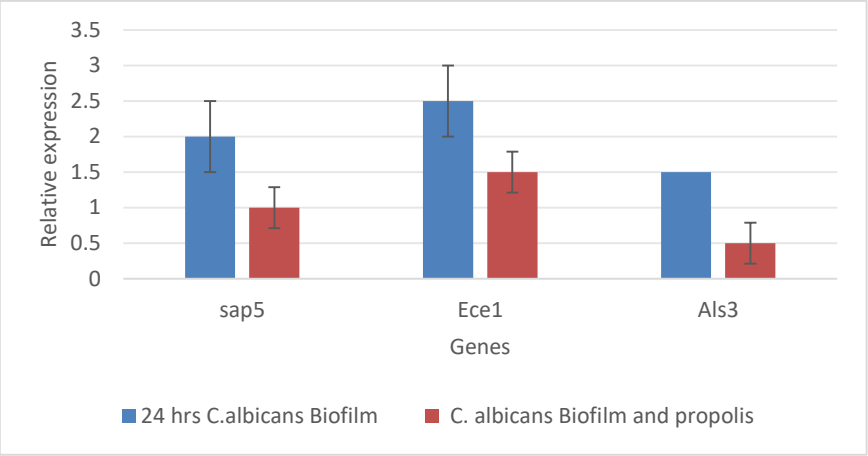

Figure (4): the relative expression of the implicated genes (*Ece1, Sap5, and Als3*) in *C. albicans* during the biofilm experiment for 24 and *C. albicans* biofilm with propolis using reverse transcription real-time PCR. (t=2.4495, df=4, standard error of difference= 0.408) p>0.05 was considered statistically nonsignificant

Results from a real-time PCR using the particular primers *Ece1*, *Sap5*, and *Als3* showed that *C. albicans* cells exposed to propolis had less biofilm formation for 48 hours. Figure 5 illustrates that propolis was applied to the *Als3* (1), *Sap5* (1.5), and *Ece1* (2) genes, respectively.

These results are conclusive evidence thatpropolis is a highly efficient inhibitor of the biofilm of *C. albicans.* It can be used as therapeutic alternatives for many diseases caused by *C. albicans*. These results have an effective effect on the genes responsible for the formation of the biofilm of *C. albicans* (**Figure 5**). The expression of the biofilm genes according to the t-test analysis was a p<0.05, considered statistically significant.

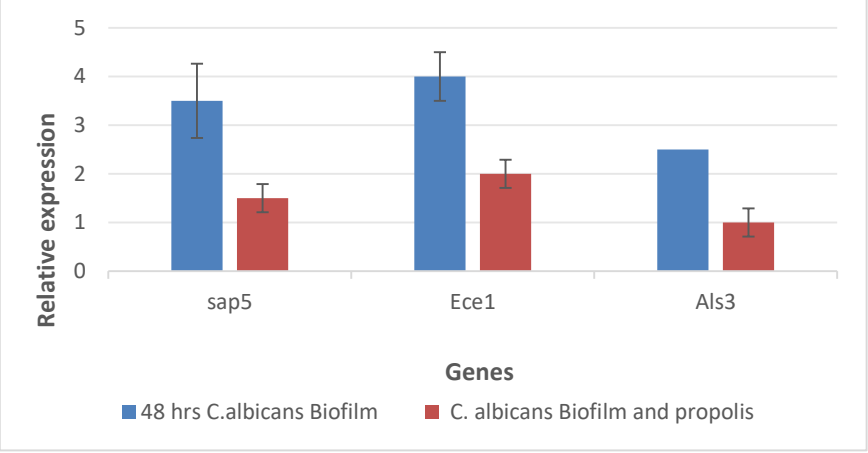

Figure (5): the relative expression of the implicated genes (*Ece1, Sap5, and Als3*) in *C. albicans* during the biofilm experiment for 48 and *C. albicans* biofilm with propolis using reverse transcription real-time PCR. (t= 3.4785, df= 4, standard error of difference= 0.527) p<0.05 was considered statistically significant

DISCUSSION

Propolis is a great natural food item with antioxidants, minerals, and simple sugars. Propolis can inhibit oxidative food deterioration processes, such as lipid oxidation in meat, fruit, and vegetable browning. Propolis inhibits the growth of microorganisms and foodborne pathogens that cause food spoilage (25, 26). Numerous studies have demonstrated the antimicrobial properties of propolis, which enable it to help treat a range of oral infections, including mucositis, ulcers, and periodontal disorders (27,28,29,30).

Studies have shown its value in treating fungal infections brought on by *Candida* species with natural remedies like propolis (31, 32). Propolis's antimicrobial activity has been studied recently as a potential substitute for traditional therapeutic approaches; however, its antifungal activity is still underappreciated, necessitating further research to fully understand its potential therapeutic application. With a MIC range of 0.185 to 3 lg/mL, an ethanolic extract of Turkish propolis exhibited the strongest antifungal activity against 76 *Candida* isolates that were isolated from the blood cultures of patients in intensive care units (33, 34, 35). On the other hand, little knowledge exists regarding propolis and its impact on *C. albicans* biofilms. This study's main objective was to ascertain whether propolis could disrupt or stop the formation of *C. albicans* biofilms. We chose to use propolis for our investigation because a variety of infections and illnesses are frequently treated with it as a folk remedy. *C. albicans* cultures were found to be successfully inhibited by a few different types of propolis, the most effective of which came from different plant origins and geographical locations. The literature emphasizes the critical role that geographic location plays in the types of propolis, primarily due to regional variations in climate and ethnobotanical flora (36). The best propolis can be found in poplar, alder, willow, elm, birch, horse chestnut, beech, and conifer tree species (37). Poplars are widely distributed throughout Europe, North America, Asia, and New Zealand. Unlike poplar propolis, which is derived from Betula verrucosa, Russian birch propolis contains both flavonols and flavones (38). Red propolis, which is found in large quantities in Brazil, Cuba, Mexico, China, and Venezuela, is derived from the plants *Dalbergia ecastophyllum, Clusia scrobiculata, Clusia minor, Clusia major*, and *Clusia rosea*. Its active phytochemicals are polyisoprenylated benzophenones (39). Similarly, the collection of Brazilian propolis is accounted for by the leaf resin of Baccharis dracunculifolia, which contains a range of phytochemicals such as higher concentrations of artepillin C, lignans, p-coumaric acid, acetophenone, and flavonoids (40). Sesquiterpenoid compounds, such as spatulenol, ledol, and germacren D, are phytochemicals that are exclusive to tropical climates. Furthermore, a type of propolis typical of the Mediterranean region can be found in Greece, Cyprus, Croatia, Egypt, Algeria, Morocco, and Malta. Its primary constituents are diterpenes, which are most likely derived from conifers in the Cupressaceae family (41, 42, 43). It was decided that propolis could be tested against *C. albicans* biofilms one more time. The development of new *C. albicans* biofilms were found to be inhibited, and existing ones were prevented by the MIC of propolis.

This study is the first to identify propolis as an inhibitor of Candida albicans biofilm genes using quantitative reverse transcriptase PCR through study propolis's MIC was 40% (w/v), and its minimum fungicidal concentration (MFC) was 50% (w/v) in biofilm-forming *C .albicans* during 24 to 48h.

The development of biofilms on the surface of yeast-form cells appears to be dependent upon adhesion genes, such as *Als3*, and their direct interaction with one another during the process (44, 45). In addition to the *Sap5* gene being found in biofilm adhered to mucosal surfaces, *Ece1* expression was associated with *Candida* hyphal elongation (46, 47, 48).

Previous research has shown that biofilm drug resistance is a complex phenomenon that is influenced by

a number of variables, including the amount of matrix formation, the makeup of the biofilm, and the simultaneous presence of bacteria in the biofilm. These variables can cause clinical problems when antifungal therapies are used (49, 50). The necessity of researching novel antifungal compounds is demonstrated by treatment failure (51). The most promising antifungal treatments seem to be natural goods like medicinal herbs (52, 53).

Propolis damages cells through a number of different mechanisms. Propolis has a reputation for harming cytoplasm and cell walls (54, 55). Nevertheless, the integrity of the cell wall is crucial for cell division. Then, the flavonoid in propolis has the ability to inhibit the process of oxidative phosphorylation, which in turn inhibits the formation of ATP. It may contribute to the inadequate growth of energy. Cell viability will thus decline (56, 57).

Limitation of the study

We limited the genes encoding the biofilm of *C. albicans* because they play a more significant role in encoding the biofilm than other genes. Also, the type of propolis used in the study was not specified, as this is the first study to determine the minimum inhibitory concentration and its effect on the genes encoding the biofilm of *C. albicans*.

CONCLUSION

According to the results, propolis's MIC was 40% (w/v), and its MFC was 50% (w/v) in biofilm-forming *C .albicans* and it can inhibit the gene expression (*Ece1*, *Sap5*, and *Als3*) *C. albicans* biofilms. Propolis has been shown to have strong antifungal properties, which implies that it may contain compounds with therapeutic value for treating infections caused by *Candida*. Whether these results can be used to treat biofilm-associated candidiasis requires additional in vivo testing.

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Conflicts of interest:

The authors declare no conflict of interest.

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التأثير العلاجي للبروبوليس ضد التعبير الجيني للأغشية الحيوية في المبيضات البيضاء

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ملخص

المادة اللزجة المسماة البروبوليس مصنوعة من النباتات وتنتجها نحل العسل .وقد استخدمت كعلاج شعبي منذ العصور القديمة، ولها فوائد صيدلانية عديدة، مثل كونها مضادة للبكتيريا والفطريات .كان الهدف من هذا العمل هو تحديد تأثير البروبوليس على التعبير عن ثلاثة جينات (Ece1و Sap5و Als3)معروفة بتورطها في تطوير الأغشية الحيوية للمبيضات البيضاء وتحديد الحد الأدنى من تركيز البروبوليس المثبط المطلوب لهذا الغرض .تم استخدام اختبار XTT لتقييم النشاط المضاد للأغشية الحيوية للبروبوليس من أجل التأكد من تكوين الأغشية الحيوية على 100عزلة من *C. albicans*من عينات البراز وحساب الحد الأدنى لتركيز البروبوليس المثبط الذي يثبط الأغشية الحيوية لـ C. albicans خلال 24و 48ساعة .أخيرًا، تم فحص تأثير البروبوليس على التعبير عن جينات Ece1و Sap5و Als3في *C. albicans*باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي ومقارنته بالنتائج التي ظهرت في التعبير الجيني للأغشية الحيوية *C. albicans*البروبوليس غير المعالج خلال 24و 48ساعة، وتم اعتباره عنصر تحكم. من خلال المقارنة، وجد أن تكوين الأغشية الحيوية ينخفض مع زيادة تركيز البروبوليس والوقت .وعليه، كان الحد الأدنى لتركيز البروبوليس المثبط 40٪ وزن /حجم، وكان الحد الأدنى لتركيزه المبيد للفطريات 50 (MFC)٪ وزن /حجم في *C. albicans*المكونة للأغشية الحيوية .بالإضافة إلى ذلك، كشف تحليل مستوى التعبير الجيني عن انخفاض في مستويات التعبير عن Ece1و Sap5و Als3مع معالجة البروبوليس خلال 24و 48ساعة.

الكلمات الدالة: التعبير، Ece1، Sap5، Als3، الجينات.

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المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

تأسست المجلة الأردنية في العلوم الصيدلانية بقرار لجنة البحث العلمي/ وزارة التعليم العالي والبحث العلمي رقم 367/2/10 تاريخ 2007/1/11 بشأن إصدار "**المجلة الأردنية في العلوم الصيدلانية**" ضمن إصدارات المجلات الأردنية الوطنية، وهي مجلة علمية عالمية متخصصة ومحكمة، وتصدر بدعم من صندوق دعم البحث العلمي والجامعة الأردنية تعنى بنشر البحوث العلمية الأصيلة المقدمة إليها للنشر في كافة مجالات العلوم الصيدلانية والعلوم الأخرى المرتبطة بها. وتصدر عن عمادة البحث العلمي وضمان الجودة في الجامعة الأردنية باسم الجامعات الأردنية كافة، خدمة للمتخصصين والباحثين والمهتمين في هذه المجالات من داخل الأردن وخارجه. وهي مجلة تصدر أربع مرات في العام أعتبارا من 2021، ومواعيد صدورها (آذار وحزيران وأيلول وكانون أول) من كل عام.

وباسمي وباسم أعضاء هيئة التحرير نود أن نشكر الزملاء الذين أسهموا بإرسال أبحاثهم إلى مجلتنا وتمكنا من إخراج العدد الأول. ونأمل من جميع الزملاء بإرسال ملاحظاتهم الإيجابية إلينا لنتمكن من النهوض بمجلتكم بالشكل الذي يليق بها.

وهذه دعوة إلى كافة الزملاء لإرسال اسهاماتهم العلمية من الأبحاث الأصيلة إلى عنوان المجلة.

والله ولي التوفيق

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