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Jordan Journal of Pharmaceutical Sciences

Aims & Scopes:

***Jordan Journal of Pharmaceutical Sciences* (*JJPS*)** is a bimonthly open-access peer reviewed journal funded by the Scientific Research Fund at Ministry of Higher Education and Research and hosted by the Deanship of Research at the University of Jordan. JJPS is dedicated to various disciplines of pharmaceutical and allied sciences. JJPS publishes manuscripts on original work, either experimental or theoretical in the following areas:

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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 pharmacoeconomic, 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 16, 2023

Letter from the Editor-in-Chief

After a full year of getting back to normal life in 2022, with all work including editorial board meetings performed face to face, the Jordan Journal of Pharmaceutical Sciences (JJPS) will continue to publish 4 issues annually at regular times i.e., quarterly, but the good news that each issue every quarter will have 15 accepted articles to be published per issue (instead of 10). The latter indicates the good achievement of JJPS last year as much more submissions were received from international countries representing 70% of total submissions while 30% were received from Jordan. Furthermore, this will decrease waiting times for researchers in receiving decisions regarding whether their submissions are either accepted or not. Also increasing the number of articles published per issue will again increase researchers' satisfaction and not delay publishing their accepted work, for example, the waiting time from receiving the submission through the decision to publishing decreased from 34 weeks in (2019-2020) to 22 weeks in (2021-2022) on average.

On the other hand, the number of citations exceeded 2 folds of the number of articles published looking forward to reaching the Q2 category in SCOPUS soon; thanks to all colleagues on the editorial board, local as well as international advisory board scientists, also special thanks to all researchers for their belief and trust in JJPS.

One important issue worth mentioning this year is the challenge of using Artificial Intelligence in writing scientific papers using new applications such as Chat GPT which since launched last November was spread not only very fast but in acceleration way all over the world. We are observing and will try to meet with all stakeholders in our field very soon to have deep discussions hoping to reach a solution to such a threat mainly in similarity percentages reports for the submissions.

In JJPS, we will continue encouraging researchers to submit their original research as well as systematic reviews and commentaries emphasizing our commitment to complete reviewing the submissions by a group of excellent scholars in a scientific logical transparent way in a short time.

Best regards

Prof Ibrahim Alabbadi
Editor-in-Chief

Editorial Commentary
Revolutionizing Healthcare through Machine Learning: Applications, Challenges, and Opportunities

Artificial intelligence powers the growing scientific field of machine learning (ML). Artificial intelligence is one of the most promising and fascinating topics of the future all over the world. ML employs statistical algorithms to enable machines to extract knowledge from large amounts of data and improve their performance on a particular task without being clearly programmed. ML has worked in a variety of areas, including health, medicine, and pharmaceuticals. These applications have the potential to revolutionize healthcare by enhancing diagnosis accuracy, drug development efficiency, interpretation of medical imaging and improving patient outcomes

Recently, medical imaging has played an important part in the diagnosis and treatment of a wide range of medical conditions, including cancer, cardiovascular, neurological, and eye diseases. An important obstacle of medical imaging is the subjectivity of image interpretation, which has a significant impact on diagnosis accuracy which, is highly reliant on personnel experience and knowledge. Machine learning algorithms can improve the accuracy as well as uniformity of medical image interpretation by detecting unique trends and characteristics in medical photographs that the human eye might not recognize. This can be performed by training machine learning algorithms on large datasets of medical images, allowing them to gain experience recognizing minor differences and abnormalities that may be indicative of disease, resulting in more precise and reliable diagnoses.

Personalized medicine is another significant application of machine learning in the health sector. The aim of personalized medicine is to customize therapies to each patient's unique characteristics, such as genetics, lifestyle, and medical history. Machine learning algorithms can assist in discovering patterns and relationships between these various variables and patient outcomes, allowing for customized and successful treatments.

Furthermore, machine learning is used in the development and validation of new drugs. Machine learning algorithms can spot patterns and relationships in large datasets of chemical structures and drug properties, allowing them to predict which drugs will be effective in clinical trials. ML can investigate pharmacovigilance investigations by analyzing huge amounts of clinical results and adverse drug reactions to find and enhance possible drug safety issues.

Despite the potential benefits of machine learning in the health sector and pharmacy science, there are still many limitations and challenges that need to be addressed. The successful implementation of machine learning in these domains necessitates thorough consideration of the ethical, social, and technical challenges associated with their use.

JJPS welcomes scientific contributions from researchers involved in this emerging discipline. We also encourage our colleagues to embrace and pursue collaborative efforts in applying machine learning and artificial intelligence to the area of health and pharmacy sciences.

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CONTENTS

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Patient Satisfaction with Pharmaceutical Services in Jordan: A Cross-Sectional Study

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ABSTRACT

Objectives: Pharmacists' responsibilities have become more patient-oriented so exploring patients' satisfaction would lead to identifying and improving the quality of pharmaceutical services. The aim of the present study was to assess patients' satisfaction with pharmaceutical services in Jordan

Methods: A cross-sectional study was conducted between December 2018 and April 2019. Patients' satisfaction was assessed using three subscales; managing therapy, interpersonal relationship, and general satisfaction, in addition, to the socio-demographic questionnaire. Participants were asked about the degree of their satisfaction with the pharmaceutical services in all subscales' questions using a 5-point Likert scale.

Results: A total of 1,333 patients participated in the study. The mean patient age was 32.4 years (SD = 11.6)52.5% (n = 700) were female. Patients had a good satisfaction score for overall pharmaceutical services with a mean score of 51.4 ± 11.4 out of 70. Among the subscales, the interpersonal relationship subscale showed a satisfaction score of 75.7% while the other two subscales (general satisfaction and managing therapy) showed slightly lower scores with 75.0% and 69.6%, respectively. Having good health and governmental or military health insurance, visiting the pharmacy more frequently, and dispensing medications from governmental hospitals, primary healthcare centers, or military hospital pharmacies were important predictors of better patient satisfaction with pharmaceutical services ($p < 0.05$).

Conclusions: This study presents some nationwide patient-reported outcomes about patient satisfaction in general pharmacy settings in Jordan. Focusing on patients' satisfaction while providing pharmaceutical services is needed.

Keywords: Patient; pharmacist; pharmaceutical services; questionnaire; satisfaction.

INTRODUCTION

The role of pharmacists was more medication-centered in the past. They focused more on the dispensing and compounding of different drugs[1]. Fortunately, nowadays, this situation has changed in an optimistic and dramatic way, and pharmacists' responsibilities have become more patient-oriented[2]. Pharmacists focus more on the interaction between them and their patients. Through this interaction and because of their responsibility to provide appropriate advice to consumers, pharmacists contribute positively by enhancing the quality of life, decreasing morbidity, and preventing and controlling so many diseases[3].

In this context, numerous new pharmacy terms have emerged such as clinical pharmacy, pharmaceutical care, and pharmacy practice. Previous studies defined "pharmaceutical care" as "The directly responsible provision of medication-related care for the purpose of achieving definite outcomes that improve patient's quality of life"[4]. A great part of pharmaceutical care is achieved during a patient's visit to the pharmacy. Proceeding from the fact that pharmacists are considered as an accessible

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resource for health and medication information due to their quick approachability and frequent contact with the public[5], many studies have emphasized on the importance behind professional communication between pharmacists and patients which may lead to optimum health care benefits[6].

Earliest studies clarified that pharmacists may engage in preventing, decreasing, or reporting medication errors, improve the patient's knowledge about the optimum use of medications, and increase patient satisfaction with various pharmaceutical services[7-10]. One way to assess the quality of pharmaceutical services is to assess patient satisfaction, which reveals whether the services are meeting the public's expectations and values or not. Patient satisfaction is the degree of positive feelings patients experience with a service. It has become an essential component of the quality of healthcare services[9,11].

Exploring patient satisfaction would lead to the identification of the healthcare sectors, including pharmacies, which need improvement. For instance, if patient is satisfied, positive effects of the healthcare organization in terms of improving patient retention rates, securing a positive local reputation, and preventing medical malpractice claims will be evident and the opposite is also true[12]. In addition, satisfaction with pharmaceutical services motivates patients to take their medications appropriately. Therefore, it is very important to conduct a study on this topic which will be helpful for pharmacy managers to understand patient satisfaction and to plan strategies to improve and advance the quality of healthcare delivery. To the best of our knowledge, there are limited studies that have explored patient satisfaction with pharmaceutical services in Jordan[13]. The most recent study investigated patient satisfaction with the medication management review service provided by pharmacists. However, the study only included two community pharmacies with a small sample size which may limit the generalizability of the study findings[14]. The main aim of the present study was to assess patient satisfaction with pharmaceutical services provided by pharmacies in different healthcare sectors (i.e. community pharmacies, hospital pharmacies, healthcare centers pharmacies) in Jordan.

MATERIAL AND METHODS

A cross-sectional questionnaire study was conducted in Jordan from December 2018 to April 2019. The ethical approval to conduct the present study was gained from Isra University (PH/1/19). The survey questionnaire tool was adapted and developed based on a previous study conducted in Spain[15]. The original questionnaire was adapted to focus on the elements of pharmaceutical services that are applicable in Jordan. The original questionnaire tool was translated into Arabic language using a forward-backward translation technique and pretested in a small sample of the general public who met the inclusion criteria as mentioned below.

The study questionnaire tool contained four main sections which were sociodemographic information in addition to three 5-point Likert subscales: managing therapy (five questions), interpersonal relationship (seven questions), and general satisfaction (two questions). The questions considered the multidimensional structure of patient satisfaction[15]. A patient's score (out of 100%) was estimated by dividing the mean score for each subscale by the maximum obtainable score for the same scale and then multiplied by 100.

Patients over 18 years of age that had visited one particular pharmacy at any region in Jordan more than once during the last three months were approached and anonymously asked to participate and complete the questionnaire using a convenience sampling technique. Completion and return of the questionnaire by the participant implied consent. Patients who participated in the study were asked about the degree of their satisfaction with the pharmaceutical services and the applicability to them of each item in the questionnaire tool using a 5-point Likert scale. Patients' responses ranged from 1 to 5, where 1 means "poor" and 5 means "excellent."

Data were analyzed using the SPSS software, version

22. The descriptive analysis was reported as mean (± SD) for quantitative variables. Categorical data were reported as percentages and frequencies. Patients' scores were interpreted as a continuous scale. Significant predictors of satisfaction toward pharmaceutical services were determined using multiple linear regression analysis. A confidence interval of 95% ($p < 0.05$) was applied to represent the statistical significance of the results and the level of significance was assigned as 5%.

RESULTS

A total of 1,500 questionnaires were distributed of which 1,333 were filled out validly (response rate 88.9%). The time needed to fill out the questionnaire ranged from 5 to 7 minutes. The mean patient age was 32.4 (SD = 11.6) years of which 52.5% (n = 700) were females, and most of the respondents had a college or university degree (n = 957, 71.8%). Table 1 shows the detailed demographic data of the study participants.

Table 1: Sociodemographic Data of the Respondents (n = 1,333)

Variable	Frequency (%)
Age (mean ± SD)	32.4 ±11.6
Gender	
Male	633 (47.5%)
Female	700 (52.5%)
Education	
High school or less	253 (19.0%)
College or Bachelor degree	957 (71.8%)
Higher degree studies	123 (9.2%)
Occupation	
Medical worker	495 (37.1%)
Non-medical worker	837 (62.8%)
Not working or retired	1 (0.1%)
Income	
Below 500 JD	731 (54.8%)
500 to 1000 JD	460 (34.5%)
1001 to 1500 JD	101 (7.6%)
More than 1500 JD	41 (3.1%)
Marital status	
Single	625 (46.9%)
Married	641 (48.1%)
Divorced	32 (2.4%)
Widowed	35 (2.6%)
Medical insurance	
No medical insurance	495 (37.1%)
Private medical insurance	356 (26.7%)
Governmental medical insurance	335 (25.1%)

Military medical insurance	147 (11.0%)
Self-reported overall health evaluation	
Good	843 (63.2%)
Fair	422 (31.7%)
Poor	68 (5.1%)

The type of pharmacy, the number of visits, and the number of medications dispensed are summarized in Table 2. The majority of participants (61.3%) visited community pharmacies more than once during the last couple of months. The mean number of visits to the same pharmacy within the last month was close to 2 (1.92 ± 1.98), and the mean number of medicines dispensed in the last visit was around 3 (2.75 ± 1.61). About 85% of participants recommended the pharmacy they dealt with to their friends and family.

Table 2: Experience of the Participants with Pharmacies (n = 1,333)

Variable	Frequency (%)
Type of pharmacy	
Community pharmacy	817 (61.3%)
Primary healthcare center pharmacy	197 (14.8%)
Governmental hospital pharmacy	150 (11.3%)
Private hospital pharmacy	87 (6.5%)
Military hospital pharmacy	82 (6.2%)
Number of visits to the pharmacy last month (mean ± SD)	1.92 ±1.98
Number of medicines dispensed in your last visit to the pharmacy (mean ± SD)	2.75 ±1.61
Did you visit other pharmacies than the one you visit always during the last month?	
Yes	467 (35.0%)
No	866 (65.0%)
Would you recommend this pharmacy which you dealt with recently to your friends and family?	
Yes	1,123 (84.2%)
No	210 (15.8%)

A total of 14 items that reflected patient satisfaction with three dimensions of pharmaceutical services were evaluated. Patients had good satisfaction scores regarding overall pharmaceutical services with a mean score of 51.4 ± 11.4 out of 70. Patients also had good satisfaction scores in the three subscales. The highest subscale was interpersonal relationship with a mean of 26.5 ± 5.5 out of 35 (75.7%), followed by general satisfaction with a mean of 7.5 ± 1.9 out of 10 (75%), and the lowest was managing therapy with a mean of 17.4 ± 4.8 out of 25 (69.6%), as seen in Table 3.

Table 3: Patient Satisfaction Score per Subscale

Subscale	Number of items	Maximum obtainable score	Mean (SD)	Patients score out of 100%§
Managing therapy	5	25	17.4 (4.8)	69.6%
Interpersonal relationship	7	35	26.5 (5.5)	75.7%
General satisfaction	2	10	7.5 (1.9)	75.0%
All scales	14	70	51.4 (11.4)	73.4%

§ Estimated by dividing the mean score by the maximum obtainable score for each sub-scale multiplied by 100.

From the subscales, the most positively evaluated item was "The courtesy and respect shown to you by the pharmacy staff" (4.1 ± 0.9; 82.0%) and then "The professional appearance of the pharmacy" (4.0 ± 0.9; 80.0%), both from interpersonal relationship. The following items recorded the lowest scores: "The information the pharmacist gives you about the proper storage of your medication" (3.2 ± 1.3; 64.0%) and "The advice you get from the pharmacist about problems that might occur with your medication" (3.3 ± 1.3; 66.0%), both from managing therapy.

Having a good health status, having governmental or military health insurance, visiting the pharmacy more frequently, and dispensing medications from governmental hospitals, primary healthcare centers, or from military hospital pharmacies were important predictors of better patient satisfaction with pharmaceutical services ($p < 0.05$) according to the results (Table 4).

Table 4: Predictors of Patient Satisfaction with Pharmaceutical Services

Variable	Simple linear regression	Multiple linear regression					
		Model 1[a]			Model 2[b]		
		B	SE	ß	B	SE	ß
Demographic data							
Age (years)	- 0.030 ; (p = 0.267)	- 0.006	0.027	- 0.007	- 0.035	0.028	- 0.035
Gender (female) (male is the reference level)	0.164 ; (p = 0.793)	0.302	0.606	0.013	0.158	0.625	0.007
Number of dispensed medicines	0.034 ; (p = 0.861)				- 0.076	0.200	- 0.011
Number of visits to the pharmacy	0.728 ; (p = 0.000)				0.754	0.159	0.131***
Type of pharmacy (community pharmacy is the reference level)							
Governmental hospital pharmacy	- 5.897 ; (p = 0.000)	- 5.368	1.056	- 0.149***			
Private hospital pharmacy	- 0.332 ; (p = 0.788)	- 0.798	1.249	- 0.017			
Military hospital pharmacy	- 11.468; (p = 0.000)	- 15.205	1.820	- 0.322***			
Primary healthcare centre pharmacy	- 3.181 ; (p = 0.000)	- 3.126	0.905	- 0.098**			
Type of insurance (no medical insurance is the reference level)							
Private insurance	0.843 ; (p = 0.280)	1.146	0.774	0.045			
Governmental insurance	- 2.874 ; (p = 0.000)	- 0.834	0.861	- 0.032			
Military insurance	- 4.467 (p = 0.000)	4.168	1.444	0.115**			

Variable	Simple linear regression	Multiple linear regression					
		Model 1[a]			Model 2[b]		
		B	SE	ß	B	SE	ß
Self-reported overall health evaluation (poor is the reference level)							
Fair	0.992 ; (p = 0.502)						
Good	3.426 ; (p = 0.016)*						
Constant		52.681	1.379		51.090	1.473	
Adjusted R^2		**0.082**					**0.015**
P-value		**0.000**					**0.000**

*p < 0.05, **p < 0.01, ***p < 0.001

a: Age, gender, medical insurance, and type of pharmacy.

b: Age, gender, number of medicines, and number of visits.

DISCUSSION

Patient satisfaction is a crucial measure of how well pharmaceutical services are offered[15]. To the best of our knowledge, the present study was unique to assess patient satisfaction with pharmaceutical services provided in different aspects in pharmacies in Jordan.

A thorough search in the literature showed that previously validated questionnaires that assessed satisfaction with pharmaceutical services were multidimensional and defined similar aspects, i.e., managing therapy, interpersonal relationship, and general satisfaction[15,16,17]. In fact, managing therapy is a vital element of pharmaceutical services since it includes items on managing drug therapy and solving therapy problems. Moreover, interpersonal relationship is also an essential component of pharmaceutical services because effective communication improves the use of medications by patients and ensures optimal therapeutic outcomes[18].

The survey results generally showed a high patient satisfaction with pharmaceutical services provided in different pharmacies types, particularly in the term of interpersonal relationships. This finding is consistent with other published studies[19,20]. The items that received high satisfaction scores were the courtesy and respect provided by the pharmacist and the professional appearance of the pharmacy. Hasan et al. (2013) showed that a pharmacist's personality, competence, and ability to reach to a patient affect consumer satisfaction[21].

Managing therapy was rated with the lowest score compared to other subscales. This confirmed the findings of previous studies that found a low satisfaction score in this subscale specifically[15,19,22], where respondents scored managing therapy items lower than they did on the interpersonal relationship and general satisfaction. Our findings revealed that the respondents were dissatisfied with a few items from this dimension specifically in providing information about proper medication storage and side effect management. This dissatisfaction may be attributed to educational factors, such as lower knowledge of antibiotics, which could contribute to the irrational use of antibiotics[23]. Similarly, Sharif et al. (2017) found in his study that only 25-30% of respondents agreed that the pharmacist explains all possible side effects and provides information on proper storage of medication[24]. These findings are not surprising since counselling patients about their medications is not always applicable, in particular in military and governmental hospitals, because of the large numbers of visitors and dispensed medications.

This study identified that higher satisfaction scores were associated with a good self-reported health status by the patient, having governmental or military insurance, having larger number of visits to the pharmacy, and dispensing medications from governmental hospitals,

primary healthcare centers, or from military hospital pharmacies. A number of published studies reported an association between patient health status and patient satisfaction[25, 26]. Xiao and Barber's study (2008) reported similar findings that higher scores were found for patient satisfaction in patients who rated their health status as excellent or good[27]. Another study by Rahmqvist reported that patients with poor health status were more likely to be dissatisfied[25]. Good satisfaction by the patients with governmental or military insurance who visited governmental centers or military hospitals could either mean that patient satisfaction received a lot of attention in these institutions or the patients were more concerned that their healthcare services would be affected if they mentioned dissatisfaction.

Regarding patient insurance, a recent report by Riffkin showed that satisfaction with healthcare among Americans is highest among patients with the military as well as governmental health insurance and is lower among those with self-paid insurance[28]. Americans with no medical insurance were the least satisfied of all patients. Similar results were found in the present study since patients with military insurance, who usually attend military hospitals, or those with governmental insurance who visit governmental hospitals and primary healthcare centers were more satisfied from pharmaceutical services than others.

The number of visits to the pharmacy was a positive predictor of patient satisfaction in the present study. Visiting the same pharmacy frequently and not going to another one could imply that the patients are feeling more comfortable and satisfied with the services offered to them in that specific pharmacy. A previous study in Palestine highlighted that around 38.8% to 49.1% of the patients reported that they visited the same pharmacy to dispense their medications or to receive the required pharmaceutical care[29]. On the other hand, a previous study from Lebanon found that patient satisfaction was positively and significantly correlated with the patient's reason for visiting the pharmacy rather than the number of visits. Patients prefer specific pharmacies over others due to different reasons such as geographic proximity, convenient working hours of the pharmacy, and the presence of trusted and qualified pharmacists and friendly staff[30].

Although participants were randomly selected from the various sectors of pharmacy and the sample size was considered good, the present study has some limitations. First, this study might be subjected to social desirability bias because the questionnaire was about pharmaceutical services provided by the pharmacy that the participant frequently visits. Second, the nature of the pharmaceutical services provided are different among different pharmacy sectors which might affect patient satisfaction.

CONCLUSION

This study presents some nationwide patient-reported satisfaction in pharmacies in Jordan. However, modification of pharmacists' professional behavior, namely in managing the therapy of patients, is necessary. This is because most patients are not made aware of drug related information, such as storage conditions and managing side effects, when a prescription is dispensed.

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Conflict of interests

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رضا المرضى عن الخدمات الصيدلانية في الأردن: دراسة مقطعية

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ملخص

المقدمة: أصبحت مسؤوليات الصيادلة أكثر توجهاً نحو المريض، لذا فإن استكشاف رضا المرضى سيؤدي إلى تحديد وتحسين جودة الخدمات الصيدلانية. كان الهدف من هذه الدراسة هو تقييم رضا المرضى عن الخدمات الصيدلانية في الأردن.

منهجية البحث: أجريت دراسة مقطعية بين ديسمبر 2018 وأبريل 2019. تم تقييم رضا المرضى باستخدام ثلاثة نطاقات فرعية؛ إدارة العلاج والعلاقة الشخصية والرضا العام، بالإضافة إلى استبيان المعلومات الديموغرافية. سُئل المشاركون عن درجة رضاهم عن الخدمات الصيدلانية في جميع أسئلة المقاييس الفرعية باستخدام مقياس ليكرت المكون من 5 نقاط.

النتائج: شارك ما مجموعه 1333 مريضا في الدراسة. كان متوسط عمر المريض 32.4 سنة (SD = 11.6) ، منها 52.5 ٪ (ن = 700) من الإناث. كان لدى المرضى درجة رضا جيدة عن الخدمات الصيدلانية الإجمالية بمتوسط درجة 51.4 ± 11.4 من 70. من بين النطاقات الفرعية، أظهر المقياس الفرعي للعلاقات الشخصية درجة رضا تبلغ 75.7٪ بينما أظهر المقياسان الفرعيان الآخران (الرضا العام وإدارة العلاج) درجات أقل قليلاً بنسبة 75.0٪ و 69.6٪ على التوالي. كانت الصحة الجيدة للأشخاص، وتوفر تأمين صحي حكومي أو عسكري، وزيارة الصيدلية بشكل متكرر، وصرف الأدوية من المستشفيات الحكومية أو مراكز الرعاية الصحية الأولية أو صيدليات المستشفيات العسكرية عوامل تنبؤية مهمة لرضا المرضى بشكل أفضل عن الخدمات الصيدلانية.($p < 0.05$)

الاستنتاجات: تقدم هذه الدراسة بعض النتائج التي أبلغ عنها المرضى على الصعيد الوطني حول رضا المرضى في البيئات الصيدلية العامة في الأردن. هناك حاجة إلى التركيز على رضا المرضى أثناء تقديم الخدمات الصيدلانية.

الكلمات الدالة: مريض، صيدلاني، خدمات صيدلانية، استبيان، رضا.

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Safety Practices in Community Pharmacy during COVID-19 Pandemic in Jordan

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ABSTRACT

Community pharmacies play a significant role in providing medicines, vaccines, consultations, and other important health services to the public. Community pharmacies continued to provide their services during the COVID-19 pandemic in most countries around the world, and this was the case in Jordan. During the COVID-19 pandemic, the pharmacy staff needs to avoid the risk of exposure to the virus causing COVID-19 along with reducing the risk for customers. This paper summarizes the safety practices of most community pharmacies in Jordan during the COVID-19 pandemic, to protect staff and customers from the risk of exposure to COVID-19 infection. Data were collected in two folds. First, a survey was distributed online through social media targeting those pharmacists working in community pharmacies. Second, face-to-face interviews were conducted with the staff and owners of pharmacies in Jordan, asking about the procedures followed to enhance the safety practices of pharmacists. Analyzing responses revealed that since the start of the pandemic, about 94% of pharmacists were using personal protective equipment, 88% of pharmacies were frequently sterilizing the pharmacy and the main door handle, and 82% of pharmacies were providing medical masks, gloves, and alcohol at the entrance.

Keywords: Community pharmacy; COVID-19; pharmacies; infection; safety considerations; Jordan.

1. INTRODUCTION

A novel coronavirus disease was identified in Wuhan, China in late December 2019, which was responsible for the new cases of pneumonia [1], [2]. This disease has spread quickly around the world [3], [4]. Implications said that the virus was transmitted from bats after a mutation in the spike glycoprotein and could infect humans [3]. This virus caused a disease that was named recently as coronavirus disease 2019 (COVID-19) [4]. Since December 2019, this highly contagious disease has been spreading rapidly around the world and increasing the number of cases and deaths [5]. The World Health Organization (WHO) has declared COVID-19 a global pandemic as most countries have registered COVID-19 cases.

COVID-19 is a highly contagious disease and is believed to be transmitted mainly by respiratory droplets [5]. The center for disease control and prevention has published some considerations for pharmacies during the COVID-19 pandemic, to reduce the risk of staff exposure to the virus along with reducing the risk for customers [14]. In addition, the WHO recommends the use of respiratory protection by using personal protective equipment (PPE). The PPE includes the use of gloves, medical masks, face shields or goggles, and gowns [15]. In specific cases, the WHO recommends the use of respirators of N95 or FFP2 standard or equivalent [15].

The COVID-19 pandemic had challenged all of the

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healthcare workforces by adding new responsibilities and roles to practitioners [12]. Pharmacists were not excluded in all countries around the world. Suddenly and with short notice, pharmacists around the world were expected to treat, immunize, test, and educate patients, within an evolving-fuzzy and uncertain clinical, health policy environment, and service delivery [12]. Pharmacists and other staff in the community pharmacy are among the first-line health care workers who might be exposed to the novel COVID-19. They have performed important responsibilities and roles to mitigate the adverse impact of COVID-19 and reduce its effects on patients [13].

Staff working in health organizations including pharmacists, nurses, physicians, and others must consider the instruction of the WHO in dealing with COVID-19 and controlling the widespread panic associated. In addition, those medical and all other medical-related students who are performing their practice in hospitals must be aware of the COVID-19 challenge to maintain a high level of safety. In Mexico, the fourth-year medical students showed a positive perspective toward a course related to patient safety and the quality of healthcare [18].

The aim of this paper is to discuss the precautions and safety considerations followed by community pharmacies in Jordan to protect staff and customers from the risk of COVID-19. Face-to-face interviews with pharmacist were conducted along with a survey was distributed through social media and targeted those pharmacist working in the community pharmacy in Jordan.

2. METHODS

Data for this paper were collected through two methods. First, a survey that was distributed online through social media targeted those pharmacists working in community pharmacy. The survey collected demographic data about participants and the implemented procedures in pharmacy to protect the staff and customers from COVID-19. Second, a face to face interviews were conducted with the staff and owners of several pharmacies in Jordan.

The objective of considering these data collection methods was to find the pharmacy's adaptation to the COVID-19 in Jordan, to protect their staff and customers from the COVID-19 risk. Several questions were asked to pharmacies' staff and owners regarding their procedures in protecting staff and customers. These questions were related to the community pharmacy itself, customers, staff, and delivery vehicle drivers who are in charge of delivering medications.

3. RESULTS AND DISCUSSION

3.1 Part 1: The Face to Face Interview

Many pharmacists who were working in community pharmacy along with pharmacy owners were interviewed. The objective was to ask about the procedures being followed to minimize the COVID-19 risk to staff and customers. The considerations being followed were related to the store, staff, customers, and delivery vehicles. The following subsections provide more details.

3.1.1 The Pharmacy-related Considerations

Several strategies have been considered by the community pharmacy to avoid direct contact between customers and staff, for reducing the risk of having COVID-19. Since COVID-19 is transmitted mainly by respiratory droplets from patients to surfaces and objects, at the early stages of the pandemic, the community pharmacy performed the following to reduce the risk of COVID-19.

(1) Having a counter of 0.9 meter of minimum width in the pharmacy divides the pharmacy into an inside area for staff and an outside area for customers. This counter was used in most pharmacies.

(2) Establishing a single line in front of the counter for the waiting customers. This was done by drawing marks/signs on the floor, either with paint, colored plaster, sticker, etc. The idea is to ensure a two-meter social distance between customers in line. For the customer at the front line, the distance between the counter and the customer's feet must be at least one meter. This ensured a minimum distance of 1.9 meter between staff and customer.

(3) At the entrance of the pharmacy, pharmacies must

provide a free hand sanitizer containing at least 60% alcohol for customer use.

(4) In the staff area, hand sanitizer and medical alcohol of at least 76% concentration must be provided in many places that are easily accessed by staff, i.e. beside the cashier, top of the counter, and other places. Pharmacy staff must have sufficient and easy access to water and soap as well.

(5) Installing a section of transparent plastic/ glass from the front-top of the splitting counter up to the pharmacy ceiling to shield against droplets from coughs and sneezes. A small window was designed at the barrier bottom for sharing items between staff and customers.

(6) Discontinuing the customer seating area and any magazines or commercial things in that area or the customer area.

(7) Cleaning the community pharmacy floor several times a day, using water and chlorine products. This should be done by making sure no customers are in the community pharmacy.

(8) Spray the shelves with disinfectant or alcohol several times a day. This included the door and its handle if it was not an auto-door. The checkout and the cashier area must be sprayed as well.

(9) Cleaning the shelves daily using disinfectant materials and wipes, in addition, to cleaning all available surfaces that could be touched by customers.

(10) Isolating a specific area in the pharmacy that was relatively far away from staff and customers, for those customers with respiratory symptoms.

(11) Stopping some of the fast-medical tests that used to be done in pharmacies such as diabetes and blood pressure tests.

(12) Hanging a large sign of the pharmacy phone number and publish this number on the pharmacy website, for encouraging customers to send their prescriptions to pharmacies or call the pharmacy to avoid paper-sharing between customers and staff.

(13) Disinfecting the plastic shield between staff and customers frequently.

(14) After licensing the COVID-19 vaccination by the American Food and Drug Administration (FDA), pharmacists were given priority with other medical staff to get the COVID-19 vaccination, as they are in the first line of dealing with COVID-19 patients.

3.1.2 The Customers-related Considerations

Customers must be aware of the risk of COVID-19. The physical contact between customers and/or staff was set to a minimum. It was recommended that all customers in pharmacies use PPE, especially medical masks and gloves. Social distances of two-meter were considered between customers and staff. Customers should not touch any surface or object unless they need to do so with care and after using the hand sensitizer. The following were the used guidelines in the Jordanian community pharmacy to avoid contagious COVID-19.

(1) Using large outdoor signs requesting customers to send the prescriptions to the pharmacy staff through some smartphone applications or by calling the pharmacy, and asking them to deliver the order home.

(2) Add large signs to assure that customers must be in line to maintain a social distance, by standing on the specific marks on the floor and they have to use the hand sanitizer located at the entrance. Some reminders from the pharmacy staff would be helpful if needed.

(3) Using signs to ask customers who have respiratory symptoms to wait in the isolated area specified for them. Staff should keep reminding customers from time to time of this.

(4) Add large signs inside the pharmacy to remind customers to not touch shelves for their protection, objects, or products, and if they did, they had to sanitize their hands. Staff should remind customers frequently.

(5) Limiting the number of customers inside the community pharmacy to prevent crowding at the checkout area.

3.1.3 The Staff-related Considerations

It is very important to staff in community pharmacies to not be infected, as this will be harmful to the

pharmacists themselves, their colleagues, and families. This was also harmful to the business, as in Jordan, and especially before approving the COVID-19 vaccines, if one of the staff was infected by COVID-19, the business was closed for 14 days. Pharmacists must use PPE including gloves, medical masks, goggles or face shields, gowns, and head caps. They must wash their hands with soap and water for a minimum of 20 seconds very frequently during the shift. They must use hand sanitizer or alcohol at least every time they touch things in the pharmacy, prescriptions, bills, or phones. The following were some guidelines that are followed in Jordanian community pharmacies.

(1) Using the PPE mentioned in the previous paragraph for personal safety.

(2) Stayed away from the customer for at least two-meter to avoid any possibility of spreading droplets through coughing or sneezing, or even talking. Stayed always at the inside counter area that was isolated by the transparent plastic sheet.

(3) Use hand sanitizer frequently after dealing with each customer especially when touching objects, currency, bills, or customer cards such as insurance and credit/debit cards.

(4) Encouraging customers to send prescriptions or needs to the pharmacy using the announced smartphone applications, which must be shown on a large sign outdoors the pharmacy or the pharmacy's website. In addition, encourages customers to call and order the medications by calling the community pharmacy and to be delivered home for free.

(5) Convince customers not to hand the insurance, debit, or credit cards to the pharmacy staff, instead, customers are encouraged to scan cards and send the image to the pharmacy email or mobile applications. For customer confidence, this image could be deleted from pharmacy phone after service in front of the customer.

(6) Gently instructing customers to be in line on the specified floor marks for standing, and for those with respiratory symptoms to stay in the specified isolation area in the pharmacy that is assigned to them.

3.1.4 The Delivery Considerations

Before the COVID-19 pandemic, medicine delivery was not permitted in Jordan by law for many reasons including but not limited to protecting the owners of single pharmacies and not allowing pharmacy chains to monopolize the market. During the COVID-19 pandemic, pharmacies in Jordan were permitted to deliver medicine under specific conditions to reduce crowdedness in pharmacies and to be in line with the lockdown strategy of the Jordanian government, to avoid the pandemic risks for people. For delivering medicines, the following were some considerations that pharmacies used for deliveries.

(1) The delivery vehicle must be sanitized on each delivery trip using suitable disinfectant and/or alcohol.

(2) The driver, who must be a pharmacist, must use all the PPE including face masks, gloves, goggles, gowns, and head caps. In addition, the driver must use sanitizer or alcohol very frequently.

(3) It is recommended that the driver does not get off the vehicle upon arriving at the delivery address, and the customer picks the order from the passenger front window. This enhances the safety of the driver.

(4) The vehicle driver, should not enter the community pharmacy, however, the driver must receive the orders with their information outside the pharmacy and the driver should not at all enter the staff area after delivering the first order.

3.2 Part 2: The Online Survey

An online survey was distributed through social media and targeted those pharmacists who are working in community pharmacy in Jordan, and especially those who used to practice and perform their jobs during the COVID-19 pandemic. The Survey collection time was two weeks during the initial stage of the COVID-19 Pandemic. A total of 170 responses were received. The survey included two main parts, the demographic parts and the part that focused on the procedures that were implemented in the community pharmacy to protect the staff and customers. A total of 17 pharmacies participated in this

survey by asking their staff to participate. The following sections describe the results obtained.

3.2.1 Results of the Online Survey

According to the responses received for the online survey, 70.6% of responses were for male pharmacists and 29.4% were for females. Based on the participants' age, 41.2% were between 20-29 years, 35.3% were between 30-39 years, and 23.5% were between 40-49 years. Based on the pharmacy educational certification, 88.2% were holding bachelor's in pharmacy or pharmacy doctor degrees, and the rest were holding diplomas in pharmacy. About 65% of participants were pharmacists working in community pharmacies and 35.3% were pharmacies owning their personal pharmacies.

Table 1 shows the percentage of the considered procedures of the community pharmacy to protect staff from the risk of infection by COVID-19. As shown in Table 1, 94.1% of pharmacies were using PPE. About 88% of them were frequently sterilizing the pharmacy area and the door handle. In addition, about 82% were providing medical masks, gloves, and alcohol at the entrance. More details are shown in Table 1.

Table 1. The considered procedures percentages of community pharmacies to protect staff against COVID-19.

The Implemented Procedure	Participants (%)
Using PPE	94.1
Installing a section of transparent plastic/ glass in front of the counter	52.9
Establishing a line in front of the counter for the waiting customers and adding marks of 1.5-2 m apart	64.7
Isolating a specific area in the pharmacy for customers with respiratory symptoms	17.6
Sterilizing the pharmacy frequently	88.2
Sterilizing the pharmacy door handle frequently	88.2
Stopping blood pressure measurement for patients	58.8
Stopping carrying out a sugar test in the pharmacy	52.9
Discontinuing the customer seating area	41.2
Asking patients to disclose if there is any symptom of corona	35.3
Providing alcohol, face masks, and gloves at the pharmacy entrance	82.4

4. Conclusions

This paper summarized the different procedures and practices that were considered in the community pharmacy in Jordan, to prevent the risk of the highly contagious COVID-19 for staff and customers. A sample of the community pharmacy was selected to perform a face-to-face interview in Jordan. In addition, an online survey was distributed through social media to collect more data about the practices being considered in the community pharmacy, to protect staff and customers from contagious COVID-19.

The main findings of the online survey were that 94.1% of pharmacists and other staff in pharmacies were using the PPE; about 88% of pharmacies were frequently sterilizing the pharmacy and the door handle. In addition, about 82% were providing medical masks, gloves, and alcohol at the entrance.

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No potential conflict of interest was reported by the author(s).

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ممارسات السلامة في صيدلية المجتمع أثناء جائحة كورونا في الأردن

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ملخص

تلعب صيدلية المجتمع دورًا مهمًا في توفير الأدوية واللقاحات والاستشارات والخدمات الصحية الهامة الأخرى للعملاء. وخلال جائحة كورونا، واصلت صيدليات المجتمع تقديم خدماتها في معظم البلدان حول العالم، ومنها الأردن. ويحتاج طاقم الصيدلية وكذلك عملائها إلى تجنب خطر التعرض للفيروس المسبب لمرض كوفيد-19 وتلخص هذه الورقة ممارسات السلامة في معظم صيدليات المجتمع في الأردن خلال جائحة كورونا، لحماية موظفيها وعملائها من خطر التعرض لعدوى الاصابة بفيروس كورونا. وقد تم جمع بيانات الدراسة في جزأين. أولاً، تم توزيع استطلاع عبر الإنترنت من خلال وسائل التواصل الاجتماعي استهدف الصيادلة العاملين في صيدليات المجتمع. ثانيًا، أجريت مقابلات وجهًا لوجه مع موظفي وأصحاب الصيدليات في الأردن، للاستفسار عن الإجراءات المتبعة لتعزيز ممارسات السلامة لدى الصيادلة. وأظهرت النتائج بأنه منذ بداية الوباء استخدم حوالي 94٪ من الصيادلة معدات الوقاية الشخصية، و88٪ من الصيدليات كانت تعقم الصيدلية ومقبض الباب الرئيسي بشكل متكرر، وايضاً 82٪ من الصيدليات قدمت الأقنعة الطبية والقفازات و الكحول للعملاء عند المدخل.

الكلمات الدالة: صيدلية المجتمع؛ كوفيد-19؛ الصيدليات؛ عدوى؛ اعتبارات السلامة؛ الأردن.

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Severinia buxifolia Leaves: Isolation, Characterization of Major Metabolites from the Bioactive Fractions and their Antiprotozoal Activity

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ABSTRACT

In an effort to explore herbal drugs as treatment for some neglected tropical diseases (NTDs), *Severinia buxifolia* (Poir) Ten, was selected and investigated for its antiprotozoal activity. Based on this objective, the ethyl acetate (EA), *n*-hexane (HE), methylene chloride (MC), *n*-butanol (BU) fractions of the leaves of *S. buxifolia* were screened for the *in vitro* antiprotozoal activity against *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania infantum*. Hexane and MC fractions showed good antiprozoal activity (IC_{50} for HE8.56, 8.64, 3.37, 10.26 µg/mL and MC 8.20, 12.7, 32.69, 2.63 µg/mL) against *T. cruzi*, *L. infantum*,*T. brucei*,and *P. falciparum* respectively. However, the EA and BU were inactive. Accordingly, two major compounds were isolated from HE identified as and friedelin (**1**) and *β*-sitosterol (**2**) while two coumarins were isolated from MC and identified as seselin (**3**) and (+)-ulopterol (**4**). Identification of the compounds was carried out based on mass spectrometry, proton and carbon 1D NMR, as well as ^{1}H-^{13}C HSQC and HMBC spectral analysis. Among isolated compounds, only seselin showed antiprotozoal activity with IC_{50} of 38.47 and 38.5 µg/mL against *T. cruzi* and *P. falciparum*, respectively and no cytotoxicity. Furthermore, an HPLC fingerprint for each fraction was achieved with the aim of authenticating the plant chemical profile and identified seslin and ulopterol as major constituents of the plant extract.

Keywords: *Severinia buxifolia* , antimalarial, antitrypanosoma, antileishmaniasis, Chinese box-orange.

INTRODUCTION

Neglected tropical diseases (NTD) tend to prevail in developing regions where water quality, sanitation and access to health care are substandard. The WHO estimates that about one-sixth of the world's population (1 billion) suffer from at least one NTD. Among NTDs are several infectious diseases caused by protozo including African sleeping sickness, leishmaniasis and Chagas' disease which are caused by *Trypanosoma brucei*, *Leishmania* spp. and *Trypanosomacruzi* spp, respectively [1]. These diseases lead to 50,000 or more morbidity and mortality per year and are primarily transmitted to humans by insects. Collectively these protozoal infections take a tremendous toll on global health because of the many disabilities associated with them that can persist for a lifetime [1].

Therapeutic regimens for controlling most NTDs are very limited and usually are not cost-effective[1]. Two drugs are used for the treatment of Chagas' disease: nifurtimox and benznidazole which are active only in the acute phase of the disease and dictate long treatment.

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Different drugs can be used in the treatment of African sleeping sickness based on the stage of the disease which includes pentamidine and suramin in the first stages and combination therapy of nifurtimox and eflornithine in later stages. Currently, leishmanesis is treated with liposomal Amphotericin-B which is known to possess serious and sometimes lethal side effects. Other treatment options of leishmanesis include pentavalent antimonials and miltefosine.

Resistance to the aforementioned drug strategies represents an urgent need to develop new generations of drugs, which should not only be more effective and safe, but also affordable and available particularly for poor communities. Traditional herbal medicines have long been used in disease control, especially in areas where NTDs are most prevalent and access to modern health facilities is limited. In this context, higher plants are thought to be a potential source of new drugsfor the control of Chagas' disease, leishmaniasis, malaria, and sleeping sickness [1,2]

Family Rutaceae (citrus family) includes many genera that are cultivated mainly for ornamentation purposes such as *Atalantia*, *Clausena*, *Murraya*, and *Swinglea* and their species. Genus *Severinia (Atlantia)* has approximately 20 species with common names such as box orange, boxthorn and Chinese box orange. *Severinia buxifolia* (Poir) Ten. (*Atalantia buxifolia*), known as boxthorn, is an evergreen short shrub native to Indochina and is cultivated as an ornamental plant [3]. It is reputed in China for its efficacy in the treatment of snake-bite, paralysis and chronic rheumatism [4]. Acridone alkaloids, coumarins, sesquiterpenoids, triterpenoids have been reported in boxthorn with different biological activities [5,6].

We have previously studied the botanical features of the plant[7] in addition to the volatile constituents of the leaf [8]. In the current study, *S. buxifolia* (Poir.) Ten. leaves ethanolic extract(95%) and its bioactive fractions were phytochemically investigated in the search for new anti-protozoal compounds.The *in vitro* activity of the isolated compounds against *Plasmodium falciparum*, *T.brucei*, *T. cruzi* and *L. infantum* was screened and their possible cytotoxicity was evaluated against human embryonic lung fibroblasts (MRC-5). Moreover, HPLC profiling for the studied fractions was performed as a chemical fingerprint of the plant.

EXPERIMENTAL

Plant Material

Leaves of *S. buxifolia* (Poir.) Ten. used in this study were collected in June 2014 from the Orman Garden, Giza, Egypt. The plant was authenticated by Dr. Mohamed El-Gebaly, senior botany specialist at the Orman Garden, Giza, Egypt. Voucher specimens (26-05-2015) were kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt.

Microorganisms:

Plasmodium falciparum, *Trypanosoma brucei*, *T. cruzi*, *Leishmania infantum* were supplied by WHO-TDR supported screening center at the Laboratory of Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical Sciences, Biomedical and Veterinary Sciences of the University of Antwerp, Belgium using the standard protocols used in WHO-TDR Drug Discovery Network.

Chemicals and Equipment

For each assay, appropriate reference drugs were used as positive control: Tamoxifen for MRC-5,(diploid human embryoinic fibroblast) Chloroquine for *P. falciparum*, Suramin for *T. b. brucei*, Benznidazole for *T. cruzi.*, Miltefosin for *L. infantum.* All reference drugs were either obtained from Sigma Chemical Co. (St Louis, MO, USA) or WHO-TDR. Silica gel H (Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 60 (70–230 mesh ASTM, Fluka, Steinheim, Germany) and silica gel (40-63µm, Fluka) were used for column chromatography. Thin-layer chromatography (TLC) was performed on silica gel GF254 pre-coated plates (E-Merck) using different solvent systems: S1**:** *n*-hexane - ethyl acetate (95: 5 v/v), S2: *n*-hexane - ethyl acetate (90:

10 v/v), S3:*n*-hexane - ethyl acetate (80: 20 v/v), S4**:** Chloroform-Methanol (95:5v/v). The chromatograms were visualized under UV and after spraying with *p*-anisaldehyde/ H_2SO_4 reagent.

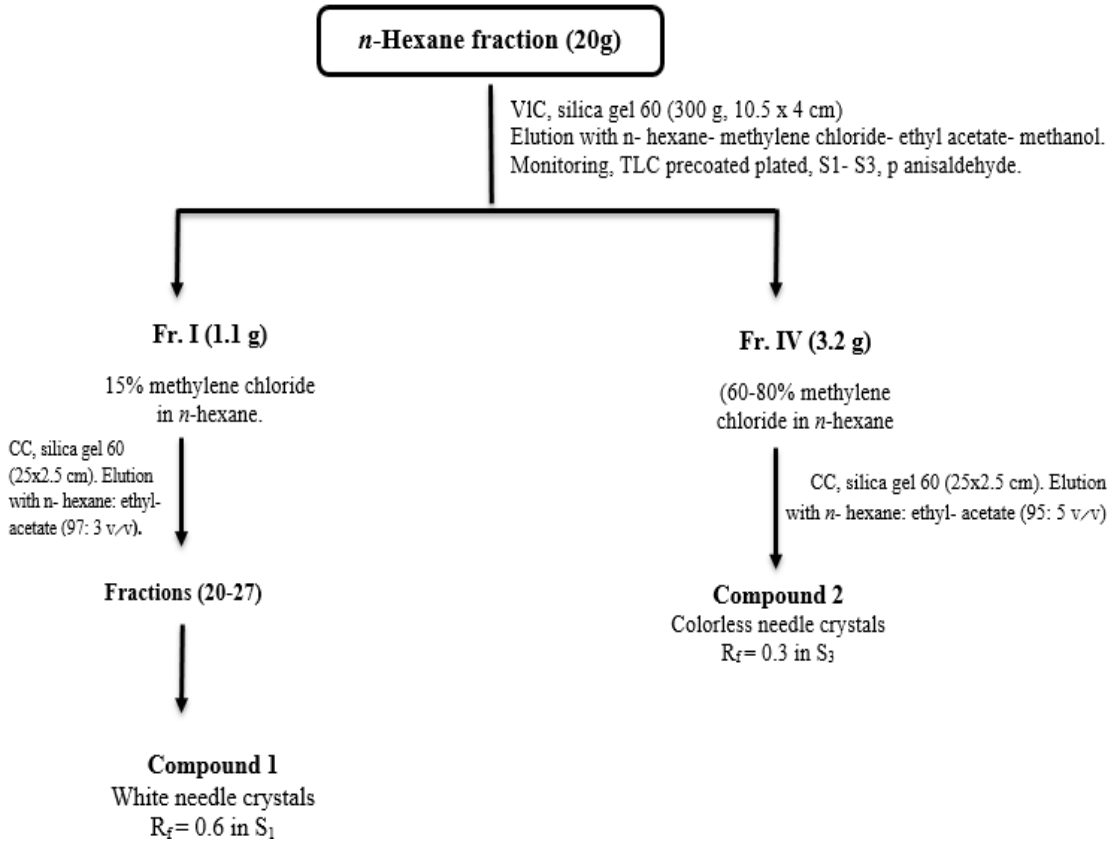
NMR experiments were performed on a Bruker Avance III NMR-spectrometer (Billerica, MA, USA). Spectral data for ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) were analyzed using Topspin 3.1 Software. NMR spectra were recorded in suitable deuterated solvents (CDCl3 or DMSO) using TMS as an internal standard and chemical shift values were expressed in δ ppm. Mass spectrometer**,** Varian Mat 711, Finnegan SS Q 7000 was used for recording mass spectra. High-performance liquid chromatography (HPLC) using reversed phase C18 column was performed using Agilent Technologies 1100 series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with a quaternary pump and degasser G1322A, series 1200, a G1314A variable wavelength detector and a G1328A manual injector. Agilent ChemStation software was used for data acquisition and processing.

Extraction, Fractionation and Isolation

The air-dried sieved powdered leaves of *S. buxifolia* (Poir) Ten. (2 Kg) were extracted with ethanol 95% (20 L) by cold maceration till exhaustion. The solvent was then removed by vacuum distillation at a temperature not exceeding 60 ºC to yield 300 gm of dry residue. The dry residue (150 g) was suspended in distilled water (1L) and partitioned successively with solvents of increasing polarities viz., *n*-hexane (400 ml x 8), methylene chloride (400 ml x 6), ethyl acetate (400 ml x 8), *n*-butanol (400 ml x 8). The solvents, in each step, were evaporated by distillation under reduced pressure to give fractions of *n*-hexane (57 g), methylene chloride (20 g), ethyl acetate (12 g), *n*-butanol (40g). All fractions were tested for biological activity.

Isolation of the major constituents of n-hexane fraction

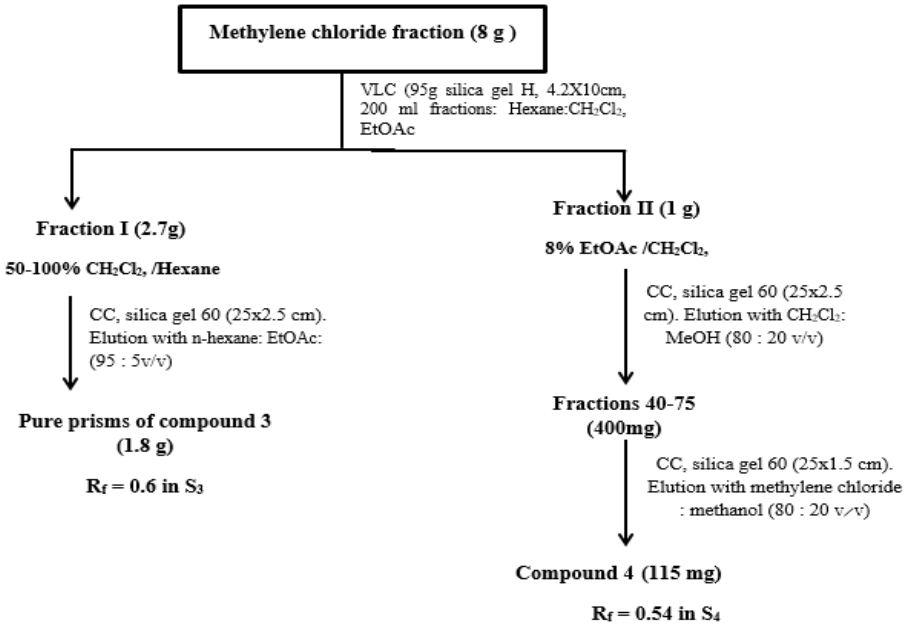
Twenty grams of the *n*-hexane fraction(HE) of the leaves were subjected to fractionation by vacuum liquid chromatography (VLC) on a 200 g silica gel 60 column (10.5 cm × 4 cm). Elution was performed starting with *n*-hexane and the polarity gradually increased by 5% by addition of CH_2Cl_2 till 100%, then stepwise addition of ethyl acetate (EtOAc) up to 50%. Fractions (200 ml, each) were collected and monitored by TLC (using S1-S3, *p*-anisaldehyde) and similar fractions were pooled. Fraction I (1.1 g): **(**15% CH_2Cl_2/*n*-hexane**)** and fraction IV were further purified yo give comprised one major spot (R_f value 0.7, *n*-hexane-EtOAC (80:20 v/v), golden yellow with *p*-anisaldehyde) was further purified on several silica gel columns (25 cm×2.5 cm) using *n*-hexane only and polarity increased gradually by ethyl acetate till 3% (97:3 v/v) to give 250 mg of white needle crystals **(compound 1)**. Fraction IV (1.32 g): **(**60-80% CH_2Cl_2-*n*- hexane), showing one major spot (R_f value 0.3, *n*-hexane-EtOAC (80:20 v/v), purplish violet with *p*-anisaldehyde) was subjected to chromatography on silica gel column (25 cm×2.5 cm) using isocratic elution with *n*-hexane: EtOAc (95:5 v/v) to finally yield 300 mg of white needle crystals **(compound 2)**, as illustrated in scheme 1.



Scheme 1: Chromatographic fractionation of the *n*-hexane fraction of *S. buxifolia* Poir.) Ten.

Isolation of the major constituents of the methylene chloride fraction of the leaves

Eight grams of the methylene chloride (MC) fraction was subjected to VLC on 150 g silica gel (10.5 cm × 4 cm). Elution was achieved using *n*-hexane 100%, 50% CH_2Cl_2 then CH_2Cl_2 (100%) then a gradual increase of EtOAc (2%). Fractions (200 ml) were collected and monitored by TLC and similar ones were pooled to afford 5 main fractions. Fraction I (1.5 g) (fractions 50% -100% CH_2Cl_2) revealed one major spot, it was further re-chromatographed on silica gel column (25 cm×2.5 cm) using *n*-hexane: EtOAc (95**:**5 v/v) to give one major pure spot, **Compound 3** (1.8 g), pale yellow prisms (R_f value 0.6, *n*-hexane-EtOAC (80:20 v/v), blue with *p*-anisaldehyde). Fraction II (1g) (8% EtOAc/CH_2Cl_2) showed one major spot ($R_f$value 0.54 in $CHCl_3$-MeOH (95:5 v/v), blue with *p*-anisaldehyde) was purified on silica gel columns (25 x2.5cm) eluted with CH_2Cl_2:MeOH (90:10 v/v) and the polarity of MeOH was gradually increased to yield one pure yellowish-white powder, **compound 4** (115 mg) as illustrated in scheme 2.



Scheme 2: Chromatographic fractionation of the methylene chloride fraction

***In vitro* Anti Protozoal Study:**

Test plate production:

Experiments were performed in 96-well plates (Greiner, Bio-One Ltd, UK), each plate containing 16 samples at 4-fold dilutions in a dose-titration range of 64 mg/mL to 0.25 mg/mL. Dilutions for compounds /fractions were carried out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Initially, two-fold serial dilutions were made in 100% DMSO to ensure complete solubility. An immediate dilution step (1/20 dilution: 10 µL compound/ fraction solution in 190 µL cell medium and test system) so that the final in-test concentration of DMSO did not exceed 1%. Each plate comprised medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and positive controls (chloroquine, miltefosine, suramin, nifurtimox for *P. falciparum, L. infantum T. brucei and T. cruzi*, respectively) [9]. All tests were run in triplicate.

Plasmodium falciparum:The chloroquine-sensitive *Plasmodium falciparum* GHA-strain was used to test plant extracts and isolated compounds according to procedures reported by Vic et al [1]. Percentage growth inhibition was calculated compared to the negative blank.

Trypanosoma brucei: Trypomastigotes of *T. brucei* Squib-427 strain (suramin-sensitive) were cultured at 37°C and 5% CO_2 in Hirumi-9 medium, supplemented with10% fetal calf serum (FCS). The assay was performed according to procedures reported by Hirumi et al [10].

T. cruzi: Tulahuen CL2 strain (nifurtimox-sensitive) was maintained on MRC-5 cells in minimal essential medium (MEM). The *in vitro* anti-trypanosomal activity was determined according to previously published procedures [11]**.** The color reaction was measured at 540 nm, values were expressed as a percentage of the blank controls.

L. infantum: Amastigotes (MHOM/MA(BE)/67) were collected from an infected donor hamster and used to infect primary peritoneal mouse macrophages (PMM). The *in vitro* anti-leishmanial activity was determined**.** Parasite burdens were determined microscopically after Giemsa staining and expressed as a percentage of the blank

controls without sample [1,2]. Additionally, cytotoxicity against PMM was assessed using the same protocol used for MRC-5 as detailed below.

Cytotoxicity assay

MRC-5 and PMM cells were separately cultivated in MEM medium, supplemented with L-glutamine (20 mM), 16.5 mM $NaHCO_3$ and 5% FCS at 37°C and 5% CO_2. For the assay, 104 MRC-5 cells/well were placed onto the test plates containing the pre-diluted samples and incubated at 37 °C and 5% CO_2 for 72 h. Cell viability was determined fluorimetrically after the addition of resazurin [2]**.** Cytotoxicity was evaluated against human embryonic lung cells (MRC-5).

HPLC Profiling for *n*-Butanol, Ethyl Acetate and Methylene Chloride Fractions

The analysis was achieved on a zobrax ODS C_{18} (particle size 5 µ.m, 250 mm x 4.6 mm) (Merck, Germany) equipped with a 5 µm, 10 x 4 mm guard column. The column temperature was maintained at 25ºC using mobile phase: solvent A (acetonitrile) and solvent B (0.3% aqueous *ortho*-phosphoric acid (v/v)). Qualitative analysis was done using a linear gradient elution system from 16% to 100% A in B for 25 minutes, at a flow rate of 1 mL/min. Injection volume and UV wavelength were set at 20µl and 310 nm respectively. Salicylic acid standard was obtained from Merck Co., Darmstadt, Germany, while standards of isoquercitrin, hyperoside, chlorogenic acid, kaempferol, quercetin were obtained from Sigma-Aldrich (Steinheim, Germany). Seselin and ulopterol reference standards used for HPLC were isolated and identified in this work. Solutions of the standards as well as the tested extracts for spiking experiments (3.5 mg / 2.5 ml MeOH).

RESULTS AND DISCUSSION

The bioactive fractions; *n*-hexane (HE) and methylene chloride (MC) were processed for the isolation and identification of their major constituents using different chromatographic techniques. Four compounds were isolated (Figure 1); compound (**1**), compound (**2**), compound**(3**) and compound (**4**). Identification of the compounds was based on physicochemical properties, mass spectrometry, ^{1}H and ^{13}C NMR and 2D HSQC and HMBC spectral analysis and comparison to published data.

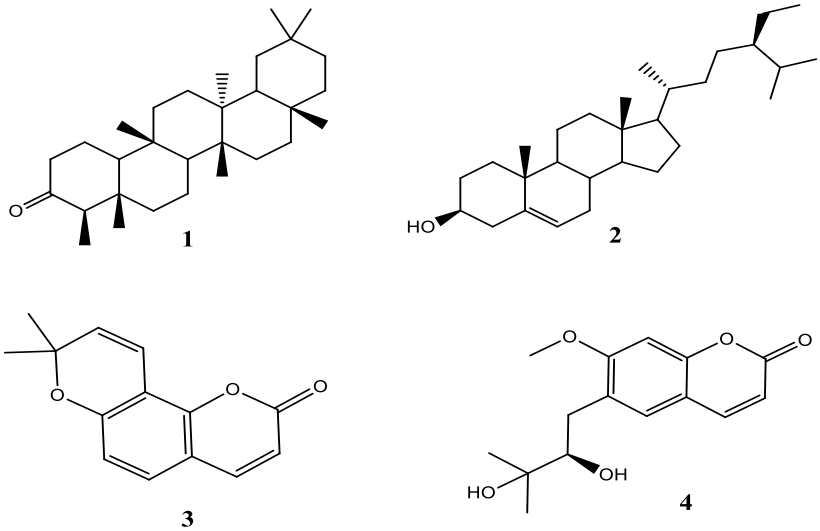


Figure 1: Structures of the isolated compounds

1: Friedelin, 2: β-Sitosterol, 3: Seselin, 4:(+)-Ulopterol

Compound 1 was isolated as white needle crystals (250 mg) from the *n*-hexane fraction. It showed a molecular weight of 426 *m/z* for $M^{+\bullet}$ corresponding to the molecular formula $C_{30}H_{50}O$ and responded positively on TLC plate when reacted with *p*-anisaldehyde/H_2SO_4 (golden yellow)**.** ^{1}H-NMR spectrum revealed the presence of seven methyl groups signals at δ_H1.1 (s, 3H, H-28), 0.98 (s, 3H,H-26), 0.94 (s, 6H,H-29,30), 0.89 (s, 3H, H-25), 0.81(d,3H,J=6.8,H-23),0.66 (s, 3H,H-24), 1.01(s, 3H,H-27) in addition to an unresolved doublet at δ_H 0.81 for eighth methyl group. All chemical shifts are characteristic of the triterpene structure**.** DEPT-Q spectrum revealed the presence of eight methyl groups resonating between 6.84 ppm and 31.8 ppm and seven quaternary carbon atoms resonating in the respective position; δ_C C-3(213.2), C-5 (42.15), C-9 (37.44), C-13(41.5), C-14(39.69), C-17(29.99), C-20 (28.17) ppm indicating possible triterpene with ketone substitution at position 3. Additionally, eleven methylene and four methine carbons were detected confirming its triterpene structure [12]. Upon comparing the results with published data, compound 1was identified as **Friedelin.**

Compound 2 was isolated and purified from the *n*-hexane fraction (300 mg)as white needle crystals**,** molecular formula $C_{29}H_{50}O$. It gave violet color in *p*-anisaldehyde/H_2SO_4indicating its steroidalnucleus. The melting point was 136-140 ˚C. ^{1}H-NMR spectrum showed the proton of H-3 as multiplet resonating at δ_H 3.59 and an olefinic proton (H-6) at 5.37(br). Signals at δ_H 1.029 (s) & 0.699 (s) corresponded to angular methyls CH_3-18 and CH_3-19 respectively [14].DEPT-Q of compound B displayed two sp^2 hybridized carbons at δ_C 146.76 and 121.72ppm, which were assigned for C_5 and C_6, respectively. DEPT-Q spectrum showed twenty-nine carbons including six methyls, eleven methylene, nine methine and three quaternary carbons. These spectral features are in good agreement with the structure of **β-sitosterol** [13].

Compound 3 was purified from the methylene chloride fraction as pale yellow prisms (1.8 gm). The compound reacted positively with both *p*-anisaldehyde/H_2SO_4 (blue color) and Dragendorff's (persistent yellow). It showed a molecular weight at *m/z* 228 for $M^{+\bullet}$ consistent with the molecular formula $C_{14}H_{12}O_3$, with melting point 119-120ºC. ^{1}H-NMR spectrum showed six doublet signals in the downfield region indicating the presence of six olefinic protons. Additionally, an upfield signal at δ_H 1.45 (s, 6H) can be assigned to two equivalent methyls. ^{13}C-NMR revealed the presence of 13 carbon signals in the molecule while DEPT-135 spectrum showed only the presence of seven positive signals and the absence of negative signals (CH_2). These seven carbons were assigned as six olefinic CH; 112.4 (C-3), 143.9 (C-4),127.8 (C-5),113.4 (C-6), 130.7 (C- 3´), 114.91(C-4´) and one signal for two equivalent CH_3 at δ_C= 28.1 (C-5´, 6´). HMBC correlations indicated a pyranocoumarin skeleton with two germinal methyls [14]. The identity of the compound was further confirmed by comparing its physical and spectral data with available literature and compound 3 was identified as **Seselin,** Figure 1.

Compound 4 was isolated from the methylene chloride fraction as a yellowish-white powder (115 mg). The compound gave a blue color with *p*-anisaldehyde/H_2SO_4. It had a molecular weight of 278 for $M^{+\bullet}$ that corresponded to the molecular formula $C_{15}H_{18}O_5$. Proton and carbon NMR spectra showed signals characteristic for coumarin molecule. Examining splitting patterns and coupling constants led to the assignment of the two doublets at 7.62 and 6.22 ppm as *o*-aromatic protons while the two singlets at 7.32 and 6.79 ppm were assigned as pyron ring protons. The presence of a dihydroxyl*iso*pentyl group was indicated by the presence of two singlets atδ_H 1.28, 1.32 ppm (3H, each), and three other signals at 2.54 ppm (1H,*dd*), 3.65 ppm (1H,*d*), 3.01 ppm (1H,*d*). Methyl protons resonating at δ_H 3.91 were assigned for 7-O-methoxy group. Comparing the results of physical and spectral data of compound **4** with the reported

spectral data [15] confirmed the identity of compound **4** as **Ulopterol,** Figure 1**.**

The results in Table (1) are represented as scores to facilitate the interpretation of IC_{50}; extracts or compounds with score 3-4 are considered active.The *n*-hexane fraction showed good anti-protozoal activity (IC_{50} 8.56 µg/mL, Sc=3) against *T. cruzi,* (IC_{50} 8.64 µg/mL, Sc=3) against *L. infantum,* (IC_{50}3.37 µg/mL, Sc=3) against *T. brucei,* (IC_{50} 10.26 µg/mL, Sc=2) against *P. falciparum.* While anti-protozoal activity of methylene chloride was reported as the following; (IC_{50} 8.20µg/mL, Sc=3) against *T. cruzi,* (IC_{50} 12.70 µg/mL, Sc=3) against *L. infantum,*(IC_{50} 32.69 µg/mL) against *T. brucei,* (IC_{50} 2.63 µg/mL, Sc=4) against *P. falciparum.* However, both *n*-hexane and methylene chloride fractions were considered cytotoxic (CC_{50}19.5 µg/mL and 10.94 µg/mL on MRC-5 cells), respectively. Meanwhile, the ethyl acetate and *n*-butanol fractions were inactive. Nevertheless, the isolated compounds (1-4) from *S. buxifolia* showed only weak inhibitory potential against all tested protozoa except for seselin which exhibited moderate activity (IC_{50}38.47 µg/mL) against *T. cruzi,* (IC_{50}38.5µg/mL) and *P. falciparum* but had an IC_{50}>64 µg/mL against other tested protozoa (Tables 1 and 2). Although seselin showed weaker antiprotozoal activity than the fractions, it was superior to the fractions as it showed no cytotoxicity (CC_{50} > 64µg/mL on MRC-5 cells).

Accordingly, *n*-hexane and methylene chloride fractions can be considered as anti-protozoal agents against all tested protozoa as WHO consideration. On the other hand, the isolated compounds (*β*-sitosterol, Friedelin and (+)-ulopterol) individually exhibited no activity, only Seselin showed moderate activity against *T. cruzi* and *P. falciparum.*This discrepancy in activity might be attributed to the synergistic effect of the many metabolites present in the crude active fractions rather than a strong anti-protozoal activity of one or two component.

The HPLC was carried out in an attempt to identify other compounds in the active & inactive fractions and to authenticate these fractions as anti-protozoal agents. Methylene chloride, ethyl acetate and *n*-butanol fractions were analyzed by reversed phase HPLC (RP-HPLC) and the isolated compounds: seselin and ulopterol were individually analyzed under the same conditions. Furthermore, chlorogenic acid, quinic acid, caffeic acid, hyperoside, quercetin,quercetrin and salicylic acid were also injected to check for their presence in any of the tested fractions. Chromatograms of the fractions and isolated compounds are shown in Figure 2. Seselin ,ulopterol and salicylic acid were detected at R_t 23.66, 9.4, 12.1 min. respectively. Seselin and ulopterol were present in both ethyl acetate and methylene chloride but not in the *n*-butanol fraction, while salicylic acid was detected only in the ethyl acetate fraction (Figure. 2). None of the other compounds was identified in any of the fractions analyzed.

CONCLUSION

The present work describes the isolation of four compounds from bioactive non-polar fractions of the leaves of *S. buxifolia*and investigate their antiprotozoal activity. HPLC analysis revealed that the two major compounds in the active MCE were seselin and ulopterol. When tested for anti-protozoal activity, both the MCE and HE fractions were active against all tested protozoa but showed cytotoxicity against MRC-5 cell lines. However, among the isolated compounds, only seselin showed moderate activity (IC_{50} 38.47 µg/mL) against *T. cruzi* and *P. falciparum* while being devoid of cytotoxicity (Table 1 CC_{50}> 64.00 µg/mL on MCR-5). Therefore, seselin may be a good lead compound for the development of anti-protozoal drugs that exhibit a good safety profile.

Table (1): *In vitro* Antiprotozoal activity of the ethanolic extract, *n*-hexane, methylene chloride and the isolated compounds of *S. buxifolia* (Poir) Ten.:

Sample/organism	MRC-5	*T. cruzi*	*L. infantum*	*T. brucei*	*Pf-K1*	PMM cytotoxicity
	IC-50 (µg/mL)					
1	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00
2	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00
3	> 64.00	**38.47**	> 64.00	> 64.00	**38.5**	> 64.00
4	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00
EE	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00
BE	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00	> 64.00
HE	**19.5**	**8.56**	**8.64**	**3.37**	**10.26**	**32.00**
MC	**10.94**	**8.20**	**12.70**	**32.69**	**2.63**	**32.00**

MRC-5 = diploid human embryonic lung fibroblast *T. brucei=Trypanosomabrucei*, *T. cruzi= Trypanosomacruzi* ,*L. infantum = Leishmania infantum*, Pf- K1= *Plasmodium falciparum* (K1 strain - asexual forms), PMM =Peripheral mouse macrophages, BE= Butanol fraction EE= ethyl acetate fraction MCE= methylene chloride fraction , 1= Friedelin,2= β-sitosterol, 3= Seselin, 4=(+)-ulopterol

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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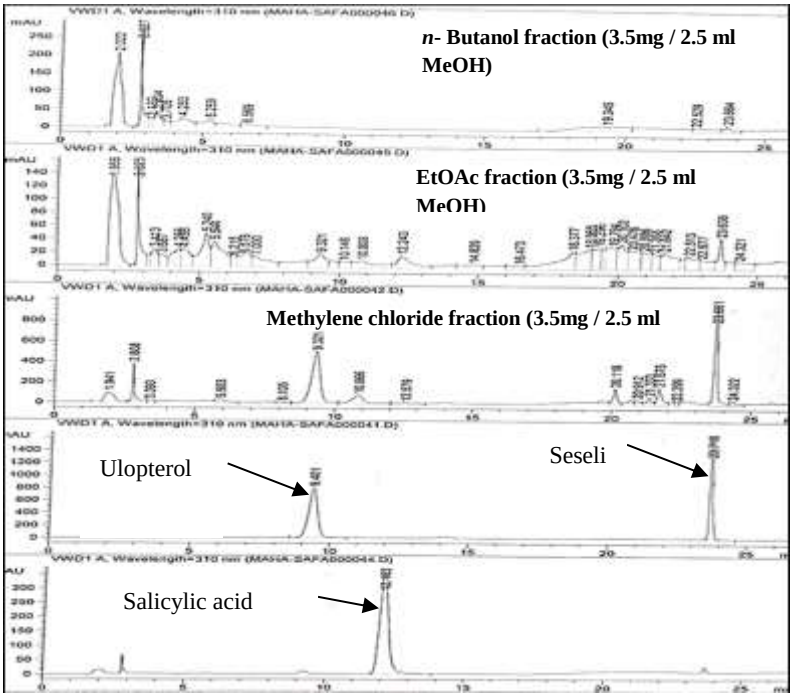

Figure 2: HPLC fingerprint of; *n*-butanol, ethyl acetate, and methylene chloride fractions, together with the major isolated compounds of the leaves of *S. buxifolia* (Poir) Ten. All extracts were prepared at concentration of 3.5mg / 2.5 ml of methanol

Table (2): Activity scores* for the antiprotozoal activity of *n*-hexane, methylene chloride fractions and the isolated compounds of *S. buxifolia* (Poir)Ten

Sample	MRC-5	*T.cruz*	*L.infantum*	*T.bruc*	Pf-K1	PMM cytotoxicity
	Activity scores					
1	< 1	= 1	< 1	< 1	= 1	< 1
2	< 1	< 1	< 1	< 1	< 1	< 1
3	< 1	< 1	< 1	< 1	< 1	< 1
4	< 1	< 1	< 1	< 1	< 1	< 1
Ex	< 1	< 1	< 1	< 1	< 1	< 1
BE	< 1	< 1	< 1	< 1	< 1	< 1
EE	< 1	< 1	< 1	< 1	< 1	< 1
HE	= 2	= *3	= *3	= *3	= 2	= 1
MC	= *3	= *3	= 2	= 1	= *4	= 1

*Score 3-4 are considered active against any protozoa Ex: total extract
PMM: Process Maturity Model (rating scale).

MRC-5 = diploid human embryonic lung fibroblast *T. brucei=Trypanosomabrucei*, *T. cruzi= Trypanosomacruzi* ,*L. infantum = Leishmania infantum*, Pf- K1= *Plasmodium falciparum* (K1 strain - asexual forms), PMM= peripheral mouse macrophages, BE= Butanol fraction EE= ethyl acetate fraction MCE= methylene chloride fraction , 1= Friedelin,2= β-sitosterol, 3= Seselin, 4=(+)-ulopterol

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أوراق سيفيرينيا بوكسفوليا: فصل وتعريف المركبات الرئيسية من المستخلاصات النشطة بيولوجيًا ونشاطها المضاد للطفيليات الأولية

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ملخص

في محاولة لاستكشاف أدوية عشبية لعلاج بعض الأمراض الاستوائية المهملة، تم اختيار نبات سيفيرينيا بوكسيفوليا(بوار تن. (العوسج الصيني) بغرض الكشف عن نشاطها المضاد للطفيليات. واستنادًا إلى هذا الهدف، تم اختبار النشاط المضاد للطفيليات لخلاصة أوراق النبات المذابة في مذيبات عضوية متعددة (الهكسان، كلوريد الميثيلي، خلات الإيثيل والبيوتانول) في المختبر خارج الجسم الحي وتحدديا ضد طفيليات *Plasmodium falciparum*.المسببة للملاريا، *Leishmania infantum*، *Trypanosoma brucei Trypanosoma cruzi* . وقد أظهرت مستخلصات الهكسان وكلوريد الميثيلين نشاطًا جيدًا مضادًا للطفيليات بمنع نمو 50% من الطفيليات عند تركيزات 8.56و8.64 و3.37 و10.26 ميكروغرام / مل لمستخلص الهكسان و 8.20و12.7 و32.69 و2.63 ميكروغرام / مل مستخلص كلوريد الميثيلين ضد طفيليات التريبانوزوما كروزي *Trypanosoma cruzi* ووالليشمانيا انفاتم *Leishmania infantum* والتريبانوزوما بروسي *Trypanosoma brucei* والبلاسموديم فالسيبارم *Plasmodium falciparum* على التوالي. بينما كان مستخلصات خلات الإيثيل والبيوتانول غير نشطة. ووفقاً لذلك، تم عزل مركبين رئيسيين من جزء الهكسان وتعرفهما باسم friedelin [**(1)**] و β-sitosterol**(2)** وكذلك تم فصل مركبين من عائلة الكومارين من جزء كلوريد الميثيلين وتم تعريفهما باسمseslin **(3)** وulopterol (+) **(4)**. وحده مركب seslin**(3)** أظهر تأثير مضاد للطفيليات وبدون سمية علي الخلايا في المختبر. تم التعرف على التركيب الكيميائي للمركبات التي تم فصلها بالإستعانة بنتائج التحليل الطيفي وإعتماد نتائج تحليل الكتلة والتحليل بالرنين المغناطيسي الأحادي والثنائي الأبعاد. وعلاوة على ذلك، تم تحليل خلاصات النبات باستخدام الكروماتوجرافيا السائلة فائقة الأداء وتحديد البصمة المعرّفة بهدف توثيق التركيب الكيميائي للنبات.

الكلمات الدالة: سيفيرينا بوكسيفوليا، مضادات الملاريا، مضادات التريبانوزوما، مضادات الليشمانيا، العوسج الصيني.

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In-process Profiling of Herbomineral Formulation from Marine Origin

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ABSTRACT

Objectives: To prepare Shankha Bhasma, an antiulcer formulation from marine origin and to standardize the formulation by two different methods of preparation and to compare with marketed formulation by modern scientific tools

Methods: Shankha Bhasma was prepared from conch shell by open and closed methods using adjuvants Kumari Swarasa and water and subjected to characterization by conventional method and AAS, XRD, EDAX, SEM, TEM, BET, TGA, IR spectroscopy. The effectiveness was checked by animal studies, toxicity by histopathology and cytotoxicity studies.

Results: Open method takes few steps and less time and shows crystalline nature (XRD).TGA shows the decomposition temp of calcium oxide. The closed method produces smoother particle (SEM,TEM and BET) with no peaks for specific organic components (IR), elements within the prescribed limits (AAS) of Ayurveda. The adjuvant's effectiveness was shown by in-process standardization and showed promising pharmacological effects without any toxic effects the dose selected was 25mg to 50mg

Conclusions: The size, smoother surface and therapeutic effectiveness of the herbominerals prepared by the two methods can be attributed to the calcination steps and adjuvants. Thus, maybe dose reduction and rapid effect can be achieved.

Keywords: Shankha Bhasma, Herbomineral, Standardization, Ayurveda, Calcination.

1 INTRODUCTION

The herbomineral formulations coming under the Rasoushadi branch of the Ayurvedic system of medicine fundamentally encompass metals and minerals as an integral part, but not in elemental form. These are normally found in form of compounds. Once in the body, they behave very differently than the elemental form of heavy metals. Huge changes occur to minerals, once they are exposed to the refined manufacturing process of Shodhana and Marana[1]. The outcome of all these methods is obtained after the treatment with several organic and inorganic herbal materials. All these exposure and methodologies may alter the toxic metal characteristics. Thus therapeutical effectiveness with high-grade safety is made possible[2]. As per the traditional physicians, the ashing process (Calcination) incorporates the depth and personality of the herbal juices in the metal[2]. The distinctive abilities of Rasausadhies like rapid action, smaller dosage, flavorlessness, lengthy shelf-life, and improved delectableness have facilitated to surmount the demands of these drugs

1.1 Incinerated Conch Shell (Shankha) - An Ayurvedic Miracle Formulations

Shankha Bhasma is prepared from the Conch (Shankha) shell of the family Gastropoda of Class Mollusca, in ash form. In Ayurveda, it is used to treat many

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diseases. Conch is known to have many properties like alkaline (kshariya), adsorbent *(grahi*) and cooling (*sheetal*). The Bhasma preparations have importance in maintaining optimum alkalinity for maintaining better health, and neutralizing harmful acids that leads to illness[3]

Since Bhasma is stable and does not metabolize, the chances of producing harmful metabolite[9]is less. It further breaks down the heavy metals present in the body. The prepared Bhasma are Micro to Nano-sized and are stable for a long period[9]. Because of their minute size, the absorption of drugs in to the circulation is fast, and thereby the dose required for producing the desired effect is small.So, Shankha Bhasma can be considered to be a better cure for stomach issues[4]. Thus, the present research work is focused on preparing Shankh Bhasma, which is an antiulcer formulation from marine source. This preparation is being done by two different methods with different adjuvants. Further, the Shankh Bhasma will be standardized and the Bhasma prepared by two methods will be compared each other and with the marketed formulation with the help of modern scientific tools.[5]

2 MATERIALS AND METHODS

2.1 Formulation of Bhasma [6-8]

Shankha was acquired and authenticated from The Government Ayurveda College Parasinikadavu, Kannur. The traditional preparation of the Shankha Bhasma was done as per the Ayurvedic Formulary of India, under Rasoushadi branch of Ayurveda. The steps involved in the preparation are [9, 10]

- Samanya Shodhana (normal purification)
- Vishesha Shodhana (special purification)
- Bhavana (heating in the sun after trituration)
- Putapaka (calcination)

The raw materials required for the preparation of the Shankha Bhasma are as follows

Table 1: Raw material used for the formulation of shankha Bhasma

S. No.	Ingredients	Quantity
1	Ashudha shankha	315 ±00g
2	Kanji	1600±00ml
3	pH of Kanji	3.4±0.2
4	Shankha after shodhana	303±0.3g
	% yield	95.87±0.64%

2.1.1 Open Method preparation of Bhasma

The calcination method used is Uthputheleekarana. The purified and triturated shankha was taken in three separate samples weighing 100gm each in a vessel known as sharava. These were then kept in a pit of coconut husk fuel and were exposed to intense heat. The process was repeated two times for all three samples (Fig.1).Changes for shankha during uthputhelekarana given in Table 2.

a b c

Figure 1: (a) Uthputhelekarana process. (b)After 1st uthputhelekarana. (c) after 2nd uthputhelekarana

Table 2: Changes for raw Shankha during open Method

Particulars	**OBSERVATIONS**	
	1st Utphulleekarana	**2nd Utphulleekarana**
Wt taken	100 g	100 g
Wt after Utphulleekarana	100 g	98.83g
Color changes	Appearance of blackish tinge	Dull white
Hardness	Became brittle	Brittle

2.1.2 Closed Method preparation of Bhasma

In the closed method, calcination was done by Puta (Repeated heating) method. Herein two adjuvants have been used.

1st Puta- Six samples each weighing 100gms of triturated Shankha were taken and kept in a Sharava. The Sharava was closed by a lid. The edges of the Sharava were sealed by a paste of mud. The three Sharava were then subjected to intense heat in Gaja Puta.

2nd Puta- Out of the six samples of triturated Shankha, three were powdered and triturated with Kumari Swarasa. These were then made in to pellets of a specific dimension Measured with the help of scale havingDiameter: 1.5 – 2 cm and thickness: 0.5 cm and dried. After drying, they were again kept in Sharava and closed by a lid. The heating process was repeated. The remaining three samples were powdered and triturated with water to make the paste and the above procedure was followed

3rd Puta- The procedure of 2nd Puta was repeated [14] Changes for raw Shankha during Closed Method have been presented in (Table: 3)

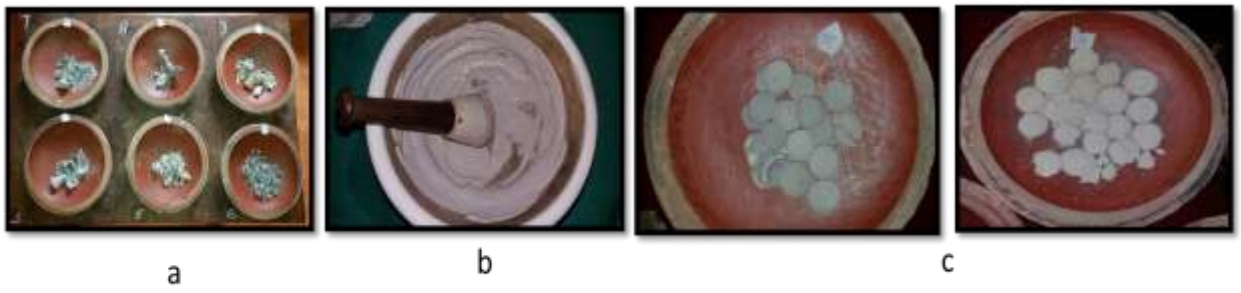

Figure 2: (a) Purified Shankha after 1st Puta (b) Trituration with adjuvants (Kumari warasa/Water) (c) Purified Shankha after 2nd Puta

Table 3: Changes for raw Shankha during Closed Method

Order of Puta	**Weight of Shankha taken**	**Weight after Puta**	**SEM**
First Puta	100 g	98.08±.584	0.238
Second Puta	98g	93.83±2.38	0.972
Third Puta	93g	89.666±4.916	2.007

2.2 Characterization of Shankha Bhasma Preparations

2.2.1 The preliminary test -are done as per traditional books.

2.2.2 Physico Chemical Standardization –

Ash Value-The inorganic residue after the heating effect removes organic matter and water is called ash. This helps in assessing the overall quantity of minerals within the drug[15.] The ash value has been determined on the based on following aspects

Total Ash- Taken a tared container, 1gm of the sample was added and heated. Heating was continued until a constant weight was obtained. The total ash was then calculated using the following formula-

$$\%\ \text{of total ash} = \frac{\text{Weight of the ash obtained}}{\text{Weight of crude drug taken}} * 100$$

Acid Ash- The total ash obtained was treated with 25 ml of dilute hydrochloric acid. It was then boiled gently for 5 minutes. After cooling, it was filtered using a filter paper. An insoluble matter was retained which was then ignited and weight was taken. The percentage of acid- insoluble ash was calculated concerning the total ash

Determination of Loss on Drying- The sample was crushed into fine powder and heated at 105° C for one hour. Loss on drying was calculated using following formula-

$$\text{Loss on Drying} = \frac{\text{Weight difference of sample}}{\text{Weight of the sample taken}} * 100$$

Solubility- Solvents of declining polarity were supplemented to definite quantity of formulation. It was found soluble in dilute HCl

Determination of pH- pH value facilitates in analysing the acidic and basic phases of adrug[16]. In the present research, the acid-basic properties of the Bhasma were tested a using shaken for 5 minutes and then allowed to settle until a clear supernatant solution was obtained. This solution was taken for valuation via DIGISUN digital pH meter

2.2.3 Instrumental Analysis-

Elemental analysis of the Bhasma by Atomic Absorption Spectroscopy- Atomic Absorption Spectroscopy is used to analyse metals at very low concentrations, typically in the parts per million (ppm) or parts per billion (ppb) ranges [16-17]. The sample was digested in dilute HCl and appropriate dilutions are made with distilled water. For conducting the spectroscopy, Varian.Make-240 has been used. Each element emits light at its characteristic wavelength, and based on the intensity of the light, the concentration of the element was calculated

IR Spectroscopy- This method is used to access the presence of organic impurities. It was carried out for the intermediates after each step of calcination and for the final preparation of Bhasma. IR spectrum was determined using Bruker FTIR spectroscopy using the ATR sampling technique. IR Spectrum of intermediate and the final product was accessed to recognize the alterations happening during the preparation of Shankha Bhasma by both open and closed methods

Thermo gravimetric curve/Differential Scanning Colorimetry-Thermo gravimetric curve has been used for checking the purity and decomposition temperature of the final products. The reduction in weight of substances has been plotted on y- axis while the temperature has been plotted along the X-axis. The process has been carried out under the atmosphere of air by exposing the sample to a controlled temperature. The test has been conducted using model FDTQ600, Make-DA instrument, USA

X-ray Diffraction (XRD)- X-ray powder diffraction (XRD) "is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions"[18]. In the present research, XRD analysis was conducted for identifying the crystalline phase and quantification of intermediate and the final preparation. The crystalline size was calculated using Scherrerequation

$$\mathbf{t = \lambda x 0.9 / \beta x \cos\theta}$$

SEM-EDAX- SEM (Scanning Electron Microscopy) has been used in the present research to study the size and morphology of particles of Shankha Bhasma. EDAX (Energy Dispersive X-ray spectroscopy) analysis was conducted to study the composition of Bhasma. These tests were carried out by using Model- EVO 18 Research SEM mode, Make-USA

BET (Brunauer, Emmett and Teller) Surface Imaging- BET analysis is used to measure surface area of a sample. It can also analyse the pore size distribution. The analysis is used to calculate the dissolution rate. Thus, it can be used to predict bioavailability[18-19]. In the present research, BET analysis was conducted using Autosorb iQ Station 1, at temperature of 77.350K

Table 4: Relative Pressure and Volume Table

Relative Pressure	Volume @ STP
1.05E-01	0.1632
1.55E-01	0.1983
2.05E-01	0.2284
2.55E-01	0.2626
3.05E-01	0.303

Transmission Electron Microscopy (TEM) - The Bhasma prepared by closed method underwent TEM analysis to assess the size of the particle[20-23]

Pharmacological Evaluation by Animal experiments- Pharmacological activity and toxicity evaluation was conducted. For this study, Wistar albino rats of both sexes were selected weighing between 150-175g. The animals were kept in polypropylene cages and food and water were given as per the requirement. The institutional Animal Ethical Committee approved the study protocols (Reg.no.1195/Re/S/08/CPCSEA). The dose was given by oral route

Acute oral toxicity study: The dose was selected from the previous study conducted by (Pandit et al.,2000) [24]. The animals were observed for 72 hours

2.2.4 Aspirin-induced antiulcer study for Shankha Bhasma- Six groups of rats containing six members in each group were selected for the study. These animals were pre-treated with a vehicle, test drug, and standard drug for 14 days by the oral route. The dose was selected from the previous study (Pandit *et al*.2000)

Table 5: Doses for Different Groups of Rats

Group 1:	1% CMC.
Group 2:	Ranitidine 50mg/kg.
Group 3:	Positive Control(Aspirin 500 mg/kg)
Group 4:	Shankha Bhasma open method 25mg/kg.
Group 5:	Shankha Bhasma open method 50mg/kg.
Group 6:	Shankha Bhasma closed method SB (b) 25mg/kg.
Group 7:	Shankha Bhasma closed method SB (b) 50mg/kg

The animals were kept fasting overnight before the study. On the day of the study, the animals were treated with Aspirin ulcerogen 500mg/kg body weight one hour after the treatment with the routine dose of the drug [25-26].

After six hours of ulcerogenic treatment, the animals were sacrificed. The abdomen was opened and the stomach was incised along the greater curvature and examined for ulcers. Ulcer lesions were counted and ulcer index was calculated.

3 ANALYSIS AND RESULTS

3.1 Formation of Shankha Bhasma- The temperature changes during each step of the preparation were noted as presented in (Table: 4).The result shows that the colour of the preparation changes from Pale white to Ash. The size of the particles was reduced by the calcination step. At high temperature, the particles get activated and the absorption is increased. This leads to reduction in the dose requirement. The assimilation of micronutrient by the added adjuvants increases the effectiveness of the Bhasma**.**

Table.6: The temperature changes during each step of the preparation

Open Method			
steps	**Time taken**	**Max.Temp attained**	**SD**
Utphuleekarana - 1st	1.15hrs	311.67±2.887	1.667
Utphuleekarana – 2nd	1.15hrs	325±50	2.887
Closed Method			
steps	**Time taken**	**Max.temp attained**	**SD**
Puta 1st	1hr	359.83±15.88	6.483
Puta 2nd	1hr	371±11.08	4.524
Puta 3rd	1hr	364.83±18.22	7.441

The values are average of three readings

3.2 Characterization of Shankha Bhasma Preparations-

Preliminary tests for standardization presented that the Shankha Bhasma prepared showed fineness. It was further found that the Bhasma is light and without any lustre and taste.

3.2.1 Physicochemical Standardization-

Physicochemical Standardization of the sample prepared by two methods has been presented in (Table: 7).

Table 7: Physicochemical Standardization of Open and Closed Methods

Physical constants	**Open Method**	**Closed method**	
Total Ash	89%	80%	82.1%
Acid insoluble ash	9.2%	8.3%	9.5%
Water-insoluble ash	15%	14%	14%
Loss on Drying	0.11%	0.09%	0.15%
pH	10.9	11.3	9.8
Solubility	Soluble in dil HCl	Soluble in dil HCl	Soluble in dil HCl
Acid neutralizing capacity	12.3mEq/gm	13mEq/gm	12.5mEq/gm

Physicochemical Standardization has been presented in Appendix 1 (Table: 8). Ash value shows the presence of carbonaceous materials in the Shankha Bhasma. The prepared Bhasma by both the method contains more than 80% ash values and loss on drying was within the limit showing that the moisture content is less in these preparations.

3.2.2 Instrumental Analysis [28–31]

Results of Atomic Absorption Spectroscopy showed that elements are present within the permissible limits. The Elemental composition of Shankha Bhasma is presented in (Table: 8).

Table 8: The Elemental composition of Shankha Bhasma

Element	Open Method	Water
Lead	2.75ppm	3.0 ppm
Copper	0.107ppm	0.721ppm
Cadmium	0.336ppm	0.299ppm
Cobalt	1.806ppm	1.223ppm
Arsenic	Within limits	Within limits
Mercury	Within limits	Within limits
Chromium	0.286ppm	0.239ppm

IR Spectroscopy by FTIR analysis of Shankha Bhasma was conducted. The peaks due to O-H stretching (3200-3600) showed the presence of moisture. Peak due to C=O stretching shown at 1603 is becoming sharp. As the Puta number is increased, C-O stretching peak is shown at 1250-1050.The absence of peaks in the functional group region indicates that the sample does not contain any major constituents. In the open method, the organic matter is getting reduced after each heating process. On comparing with raw Shankha and the peak due to C=O stretching is prominent in the final sample. In the closed method, as the number of Puta was increased, the peak due to functional group are getting diminished and C=O stretching peak became more clear. The Extra peaks shown in (b) sample are due to the added micronutrients from the adjuant used.

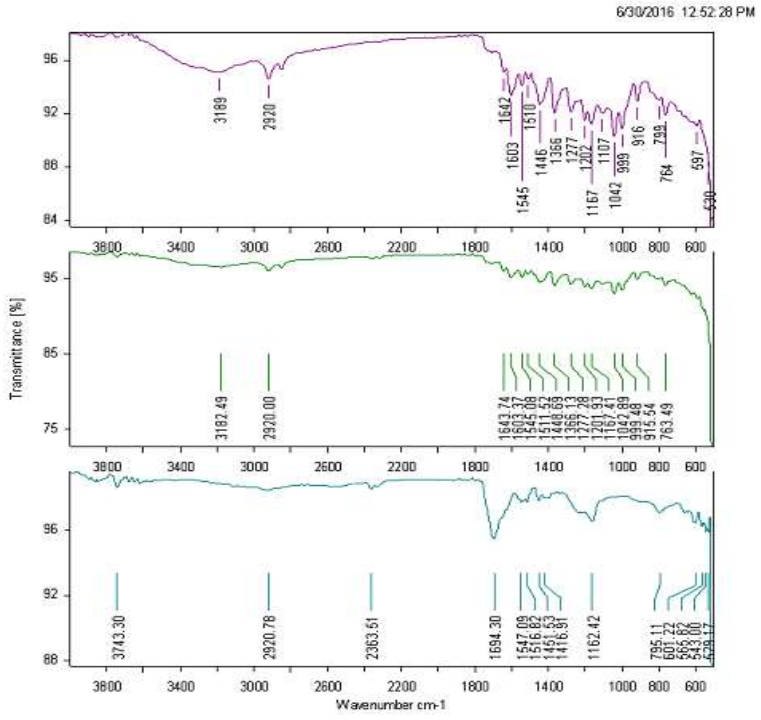

Figure 3: IR Spectrum for Shankha raw Shankha after 1st and 2nd Uthputhelekarana by Open method

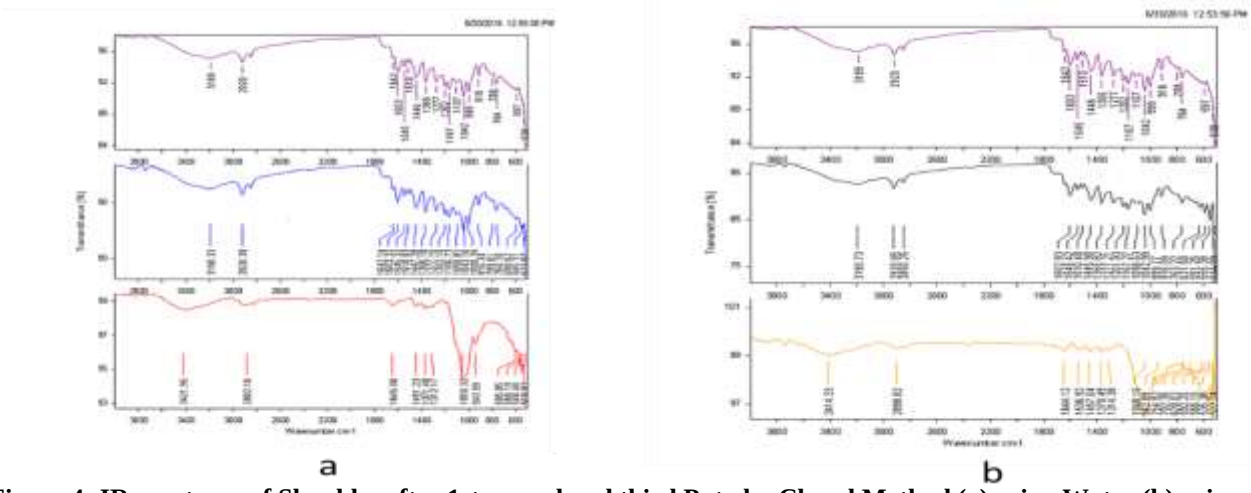

Figure 4: IR spectrum of Shankha after 1st second and third Puta by Closed Method (a) using Water (b) using Kumari Swarasa

In the TGA/DSC method, the decomposition temperature was shown as 734.5°C for the open method and 731.2°C, 729.9°C for the closed method with Kumari Swarasa and water respectively. The analysis shows the purity of the compound. This temperature is close to the decomposition temperature of calcium carbonate to calcium oxide. Both the methods produced pure calcium oxide in the final product.

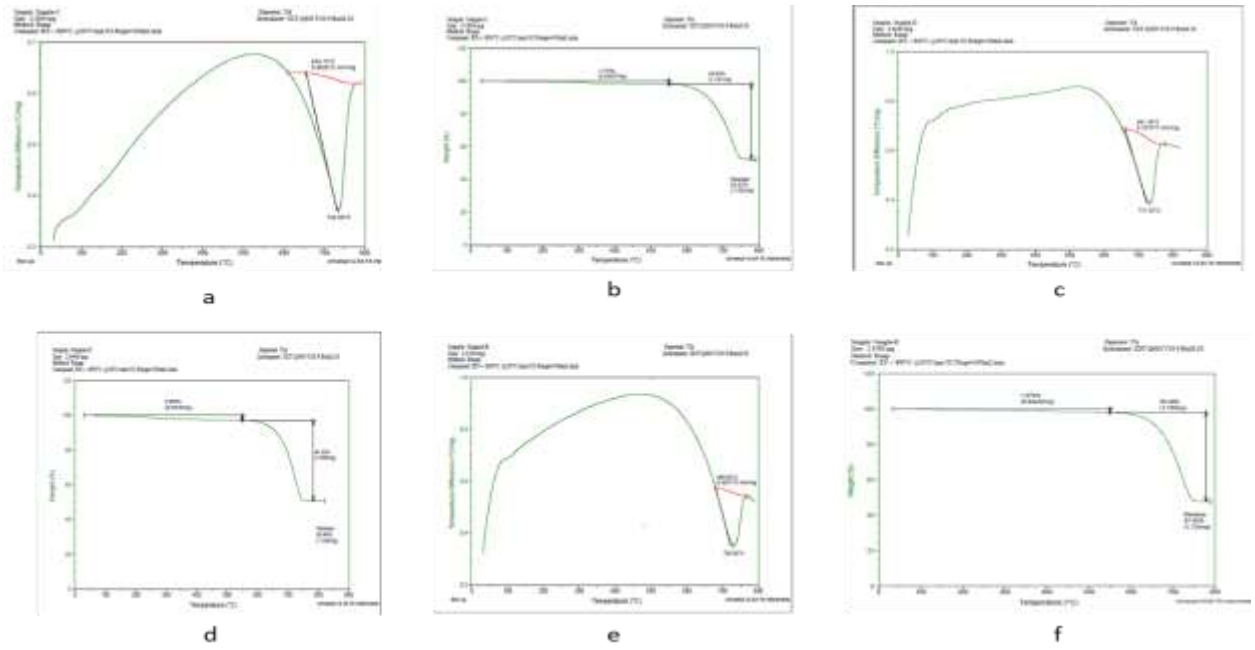

Figure 5: TGA and DSC analysis for Shankha Bhasma (a) & (b) Open method (c) & (d) Closed method with Kumari Swarasa; (e) & (f) Closed method with water

The images of the SEM analysis of Shankha Bhasma revealed that the size of the particle is reduced by the preparation technique. SEM analysis has done to determine the surface morphology and shape of particles. In the open method the temperature attained during heating process is 311ºC after first uthputheleekarana(Calcination in open condition) and 325ºC after second uthputheleekarana (Second calcination).SEM photographs shows that the particle has changed to more fine and smooth after second calcination of Open method. The surface of the particles was smooth and clustered. In Closed method the effect of heating with the adjuvants water and kumariswarasa on particle size and surface were evaluated. Closed method with water shows smoother and clearer particles than with open method .With kumariswarasa adjuvant more smooth and finer particles are produced .The heating temperature reached in closed method after Puta (calcination in closed vessal) is 359ºC - 365ºC.The high temperature attained in closed method may be attributed to more fineness of particles.

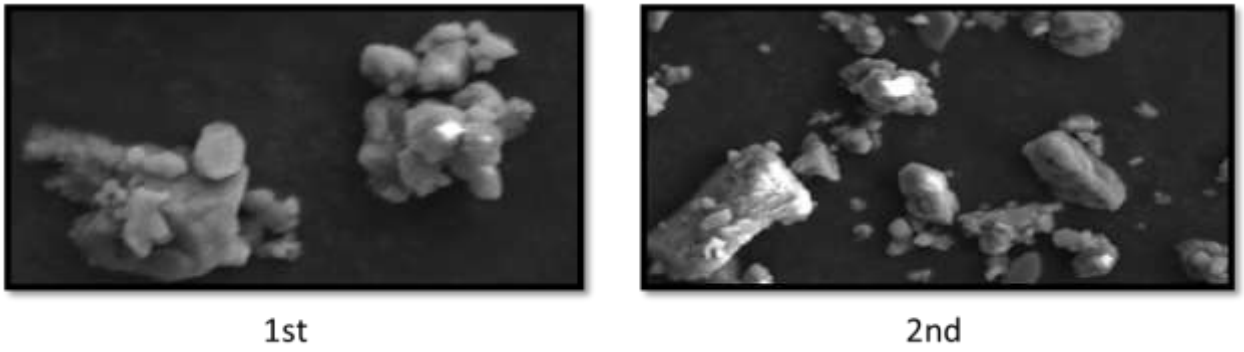

Figure 6: SEM Images for Open Method

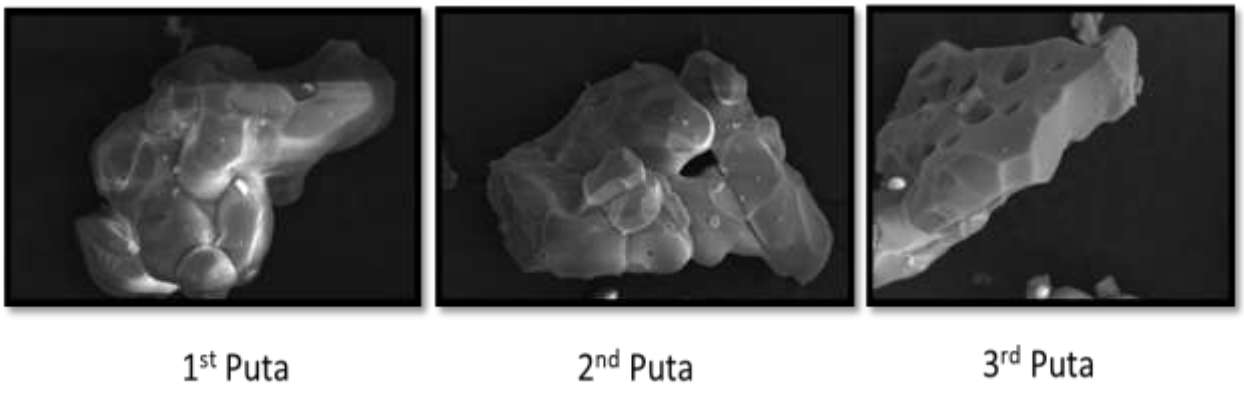

Figure 7: SEM Images for Closed Method with Water

Figure 8: SEM images for closed method with Kumari Swarasa

It can be inferred from the SEM images Fig.6, Fig.7 and Fig 8, that the heating process in the closed method makes the particle surface smooth and fluffy and in the open method the particles appear in clusters.

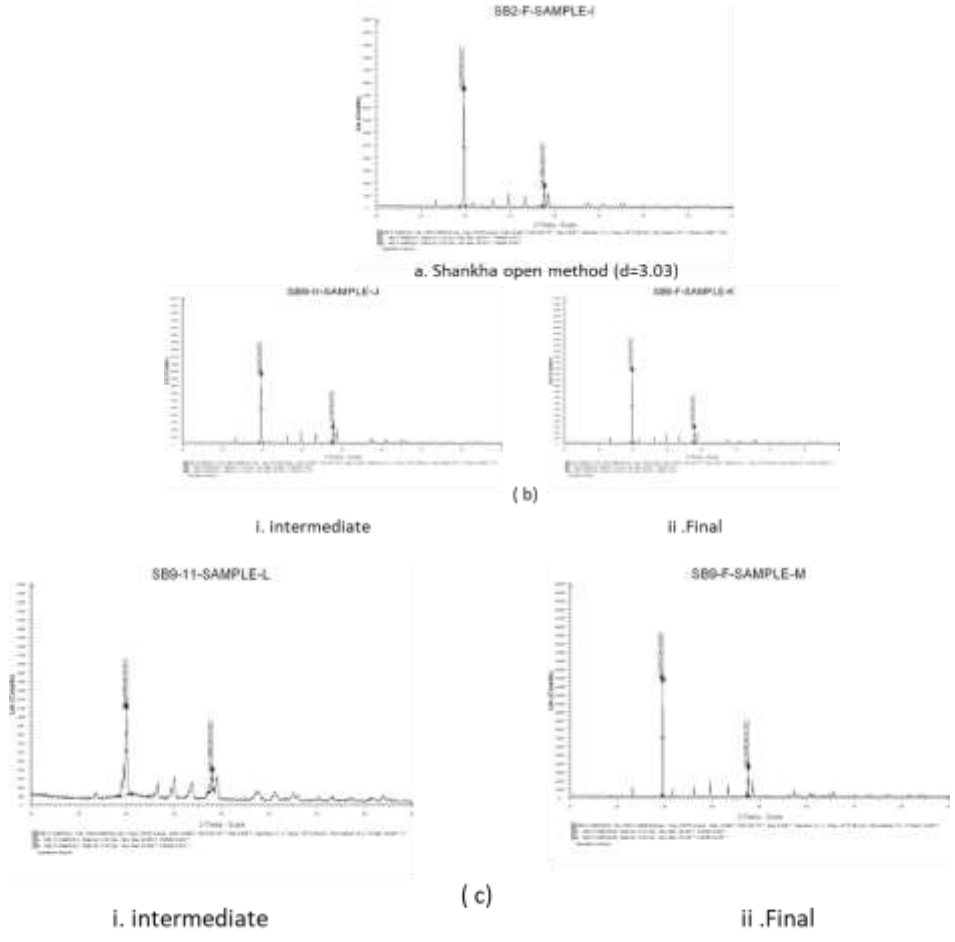

Figure 9: XRD analysis for Shankha Bhasma; b. Shankha Closed method with water (d=3.01); c. Shankha closed method with Kumari Swarasa (d=3.026)

The sharp peaks in XRD analysis (Figure 09) shows the crystalline nature of the powder and the d value was calculated using Scherrer formula. The low intensity peaks were due to the presence of trace elements or due to the micronutrients. During the preparation of Bhasma, the amorphous nature of the material is changed to crystalline nature. Both the methods produced crystals with comparable d values.

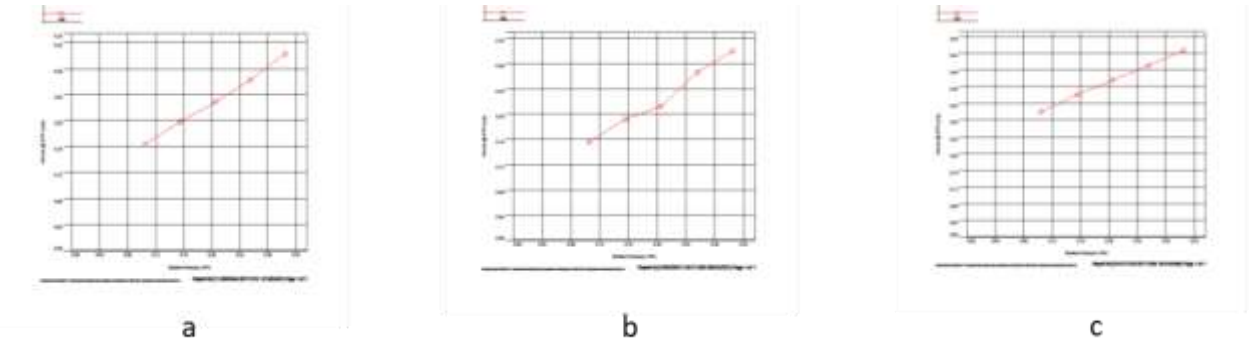

Figure 10: BET Surface Imaging (a) Open Method (b) Closed Method with Kumari Swarasa and (c) Closed Method with Water

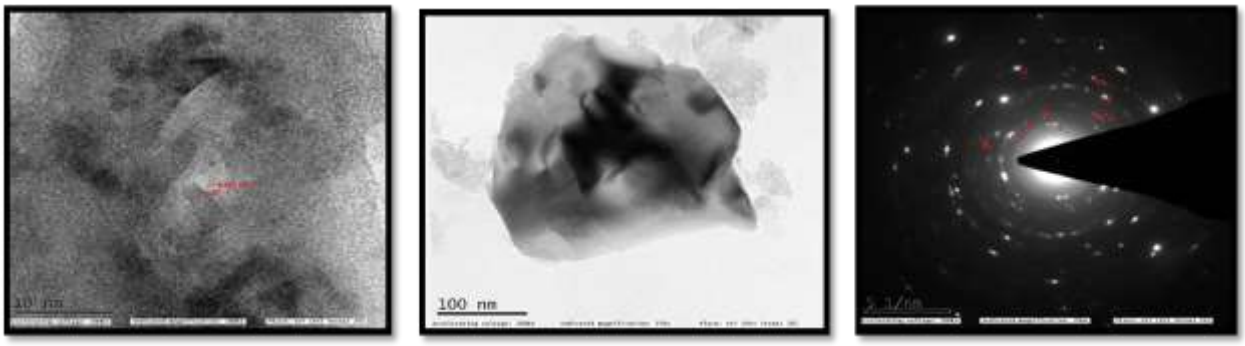

Figure 11: Particle characterization using Transmission Electron Microscopy (TEM)- Closed method

BET (Brunauer–Emmett–Teller)surface imaging of the shankha bhasma formulations(Figure 10) showed that the particle size is smaller for closed method than the open method formulation. To confirm the crystalline nature and nano size the shankha formulations prepared by closed method were characterized by TEM Analysis.TEM analysis of the closed method for preparation of Shankha Bhasma showed results presented in Appendix 1 (Table: 9).The results shows the d spacing and the number of spots indicating that the particles are of nanorange.

Table 9: TEM analysis of closed method

Spot#	d-Spacing (nm)	Rec. Pos.(1/nm)	Degrees to Spot 1	Degrees to x-axis	Amplitude
1	0.3928	2.546	0	92.16	3364.67
2	0.3317	3.015	46.8	138.97	1090.06
3	0.3089	3.238	33.58	125.74	1368.68

Spot#	d-Spacing (nm)	Rec. Pos.(1/nm)	Degrees to Spot 1	Degrees to x-axis	Amplitude
4	0.2874	3.479	10.72	102.89	1011.31
5	0.2524	3.963	35.32	56.84	1372.83
6	0.2311	4.327	44.43	47.73	1433.02
7	0.2318	4.314	71.63	163.79	1109.78
8	0.2116	4.725	70.25	162.42	665.66
9	0.191	5.235	34.27	57.89	6725.28
10	0.1645	6.078	22.72	69.44	1754.82
11	0.1471	6.799	17.85	110.01	6359.89

3.2.3 Pharmacological Evaluation- Shankha Bhasma was prepared by two different methods which were then analyzed for its pharmacological activities. The acute toxicity studies were carried as per OECD 425 guidelines for fixing the dose and checking the toxicity of the formulations under study. The Bhasma preparations were found to be safe up to a maximum dose of 2000 mg/kg body weight in acute toxicity studies. Aspirin induced method was selected for antiulcer study. The control group, standard ranitidine treated and the formulation treated animals were sacrificed and the ulcer index were calculated. Shankha Bhasma demonstrated significant protection in Aspirin induced ulcers by ulcer index calculation ($p< 0.001$) (Table10). The results suggest that this Ayurvedic preparation possess significant gastro protective activity in lower doses of the therapeutic range. Shankha Bhasma prepared by the Open method and Closed method were showed marked antiulcer activity.Open method has got reduced steps without adding any additional ingredients and finishes within a short time. While Method II (Closed method) has used two different adjuvants namely water and Kumari Swarasa (aloe vera juice) for the tricturation and Puta (calcination)The thiobarbituric acid reacting substances(TBARS) in gastric tissue is determined to assess the free radical scavenging activity and lipid peroxidation. The reduction in TBARS value for the Bhasma formulation shows that there exits a free radical scavenging activity their by gastric mucosa protection. Serum calcium level is also determined and it shows that there is no change in serum calcium leval although their exits reduction in TBARS value and thereby antiperoxidative effect. The slightly increased activity produced by Closed method shankha bhasma may be attributed to the adjuvants added. [Prasanta Kumar Sarkar,*et.al*.,2010]. The adjuvant used in closed method,Kumari swarasa itself has got wound healing activity which may be an added advantage for antiulcer effect.

The histopathological studies were done for toxicological purpose. The histopathological results in the figure below revealed the normal structure of cells of the stomach, liver and kidney and the structure of cells after administration of Shankha Bhasma. A detailed histopathological examination was carried out for the liver and kidney. The liver sections of the normal group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein. Disarrangement of normal hepatic cells with cellular necrosis, Congested portal veins, vacuolization of cytoplasm and fatty degeneration was observed in Aspirin treated rats. Kidney Sections of the control group showed normal renal glomeruli, collecting tubules intestinal tissues and internal structures whereas aspirin treated rats showed infiltrated cellular structure and slightly congested blood vessels. The liver sections of the group 4 and 6 rats treated with SB (a) Method I and SB (b) method II at the dose of 25 mg/kg showed a sign of significant protection as it was evident by

the low accumulation of fatty lobules, absence of necrosis and vacuoles in a dose dependent manner which is shown in Figure 12 below. Almost similar sign of protection was shown in the liver sections of ranitidine at a dose of 50 mg/kg treated rat group.[32-34]

Table 10: Aspirin induced antiulcer study shankha for closed and open method

S.No	Groups	Dose (mg/kg)	Ulcer Index	SerumCalcium	TBARS (nmol/mg protein)
1	Control	1% CMC	55.80±.702	9.66±0.153	14.10±0.185
2	Ranitidine	20mg/kg	19.50±.363	10.43±0.199	9.91±0.028
3	Shankha test (open method)	25mg/kg	13.78±0.631	10.25±0.631*	8.80±0.401
4	Shankha test (openmethod)	50mg/kg	10.03±0.176	10.29±0.327*	8.29±0.344
5	Shankha test (closed method)	25mg/kg	14.08±0.135	10.65±0.221	9.83±0.370
6	Shankha test (closed method)	50mg/kg	10.16±0.158	10.81±0.3446	8.71±0.380

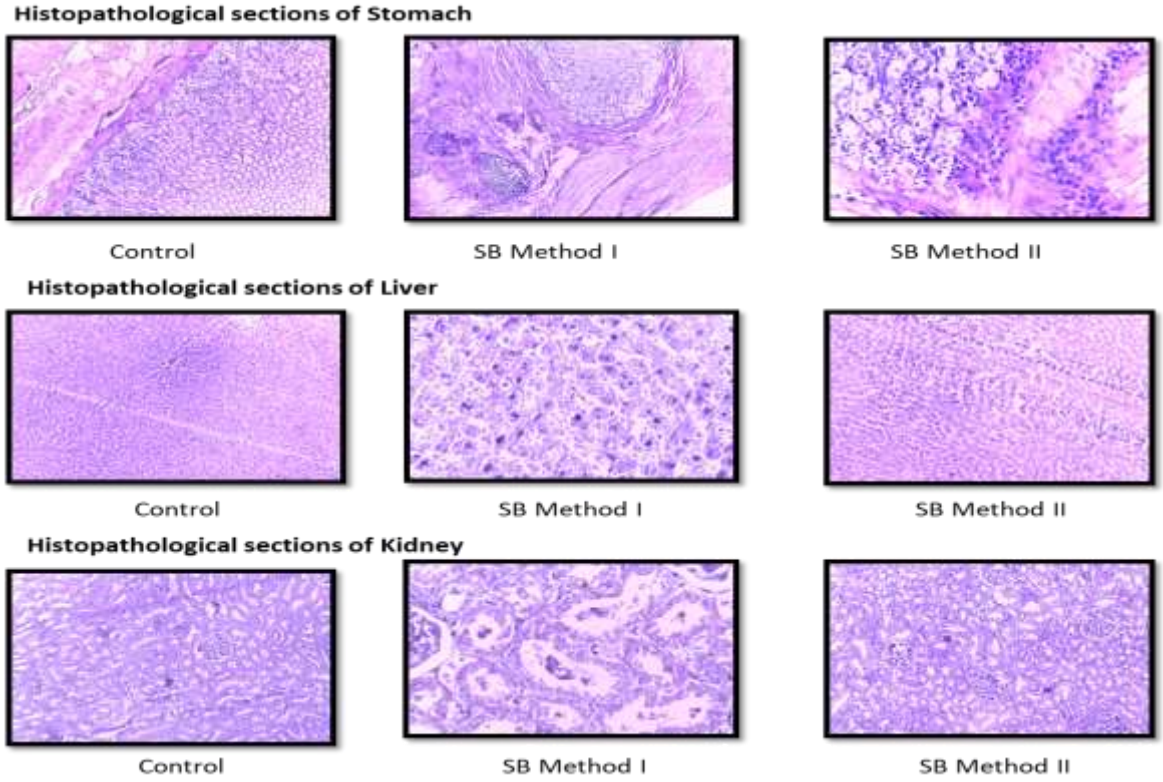

Figure 12: Histopathological images of Method I (Closed Method) and Method II(Open Method)

4 CONCLUSION

Shankha Bhasma, the herbomineral formulation prepared from the shell of marine organism contains calcium carbonate as the major component. The in process standardization for the formulation of Bhasma are done by adopting two methods that incorporates different adjuvants and which may influence, the particle size, efficacy and dose. The peculiar method of formulation technique involving Shodhana, Bhavana and Puta was used which reduced the particle size to Nano size. The method I involves heating directly, has the advantage of reduced steps without adding any additional ingredients and finishes within a short time. On the other hand, method II used two different adjuvants namely water and Kumari Swarasa (aloe vera juice) for the tricturation and Puta (calcination). It was repeated four times. The physicochemical evaluation gives the absence of carbonaceous matter and alkalinity of shankha bhasma. The evaluation parameters by AAS, XRD, TGA, SEM, TEM revealed that although the particle size and chemical constituents are almost same for two methods, the crystalline nature, surface morphology and pharmacological effect is comparatively good for method II when compared with method I. This effectiveness may be attributed to the adjuvant added and the repeated heating process. Cytotoxic studies revealed that the toxicity of this particular Shankha Bhasma prepared on stomach cell was nil and does not have any toxic effect in liver and kidney cell.

Shankha Bhasma has evaluated by WHO Guidelines and the quality control Evaluation has done for in process and for final product. A standardization parameter for the Shankha Bhasma has been established and the method can be included in the official monograph.

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في عملية تحديد ملامح صياغة هيربومينرال من أصل بحري

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ملخص

يستخدم النظام الأيورفيدا مشتقات نباتية وحيوانية لقيمها الطبية. وجود الآثار الجانبية للأدوية المتاحة لقرحة المعدة أدّى لتعزيز وزيادة الميول لإستخدام الطرق التقليدية للعلاج .

الهدف: لإعداد شانخا بهاسما (Shankha Bhasma) ، وهي صياغة مضادّة للقرحة من أصل بحري بواسطة طريقتين مختلفتين ومقارنتها بالأدوات العلمية الحديثة.

الأساليب: تم إعداد شانخا بهاسما من قوقعة المحارة بطرق مفتوحة ومغلقة باستخدام المواد المكملة (adjuvants) مثل كوماري سواراسا (Kumari Swarasa) والماء وتتعرض للتوصيف بالطريقة التقليدية و AAS و XRD و EDAX و SEM و TEM و BET و TGA والتحليل الطيفي .IR تم تحقق الفعالية من خلال الدراسات الحيوانية، وتحقق السّمّيّة بواسطة الدراسات الهستوباثولاجيا والسّمّيّة الخلويّة.

النتائج: الأسلوب المفتوح يأخذ خطوات قليلة وأقل من الوقت ويظهر الطبيعة البلورية TGA .(XRD) يظهر درجة الحرارة التحلل من أكسيد الكالسيوم. الأسلوب المغلق ينتج جسيمات أكثر سلاسةSEM) ، TEMو (BET مع عدم وجود مواد عضوية(IR) ، مع عناصر ضمن الحدود المنصوص عليها (AAS) من الأيورفيدا. وقد أظهرت فعالية المواد المكملة (adjuvants) من خلال توحيد العمليات وحصل على التأثير الدوائي الكافي دون أي تأثير سامّ.

الاستنتاج: الحجم والسطح الأكثر سلاسة والفعالية العلاجية لهيربومينرال (herbominerals) التي أعدتها من خلال الطريقتان يمكن أن يعزي إلى خطوات التكليس و المواد المكملة. وبالتالي يمكن تحقّق خفض الجرعة و تأثير سريع.

الكلمات الدالة: شانخا بهاسما، هيربومينرال، التوحيد القياسي، الأيورفيدا، التكليس.

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Anxiolytic Effect of Ethanolic Extract of Medjool Dates of *Phoenix Dactylifera* in Mice

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ABSTRACT

Background: Anxiety is one of the most frequent psychiatric disorders, affecting 33.7% of the general population. However, the consumption of healthy diets has been found to help, at least in part, in the prevention and treatment of anxiety-like disorders.

Methods: In this study, the anxiety behaviors of mice subjected to chronic intake of low-and high doses of ethanolic extract of Medjool dates (Phoenix Dactylifera) were evaluated in comparison to the counterparts of control mice. The elevated zero maze (EZM) test and marbles burying test were used as models of choice for evaluating anxiety behaviors in these mice. In addition, aphytochemical analysis of major secondary metabolite groups was done.

Results: The findings of this study revealed that the ethanolic extract of dates is rich in flavonoids and steroids with known activity as anxiolytics, such as kaempherol. Mice received a low dose (300 mg/kg) of the extract exhibited lower anxiety in the EZM than the untreated mice (negative control), which was determined by a significant increase in the latency to the closed area, a significant decrease in the time spent in the closed area and a significant increase in the number of the entries to the open quadrants. The anxiolytic effect of low dose extract was comparable to that produced in positive control mice treated with diazepam (1.5 mg/kg, i.p) in all tested parameters. Data obtained from the marble burying test also showed a significant anxiolytic effect by low dose (300 mg/kg) of the extract as compared to untreated mice, which was manifested by significant decrease in the total number of buried marbles. The anxiolytic effect of low dose extract in the marbles burying test was comparable to that produced in counterparts of positive control mice treated with fluoxetine (5mg/kg, i.p). On the other hand, chronic intake of high doses (2583 mg/kg) of the extract did not cause any significant anxiolytic effect in the EZM and marbles burying tests.

Conclusions: Overall, these results indicate that regular intake of low dose of ethanolic extract of Medjool dates may help to prevent and manage anxiety disorders. However, further studies are recommended to elucidate the putative mechanism underlying the anxiolytic effect of these dates.

Keywords: Anxiety, elevated zero mazes, *Phoenix dactylifera*, marbles burying, Medjool dates.

INTRODUCTION

Anxiety is a normal body emotion that aids humans in responding effectively to potentially dangerous or unpleasant situations.[1] However, people with anxiety disorders experience overwhelming and persistent fears that affect their everyday activities and disturb their social interactions.[2] Anxiety is a crucial component in the developing of cardiovascular diseases and has been frequently associated with other risk factors, such as depression, substance abuse, obesity and sleep disorders. [3-6] Despite the fact that the

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etiology of anxiety disorders remains unclear, it is thought to be linked to a variety of genetic, neurochemical, and metabolic factors.[7] According to data acquired from large population-based surveys, anxiety illness affects up to 33.7% of the population at some point in their lives.[8] Anxiety and Depression Association of America has envisaged that anxiety disorders are the most common mental illness in the United States, impacting 18.1 % of the population each year.[8] Examples of anxiety disorders include generalized anxiety disorder, social anxiety disorder (social phobia), specific phobias, separation anxiety disorder and obsessive-compulsive disorders.[9] The symptoms of anxiety might start as early as childhood or adolescence and last into adulthood.[10] When the symptoms become overwhelming or counseling becomes ineffective, pharmacotherapy may be considered to assist patients in coping with their anxiety. During the last decades, treatment options for anxiety disorders have expanded to include both prescription drugs and natural remedies.[8] Given that anxiety disorders are associated with a subset of underlying metabolic disturbances, that are influenced by lifestyle factors, complementary nutritional interventions for anxiety appear to be effective.[11] Several studies demonstrated that dietary phytochemicals such as phenolics, carotenoids, and dietary fibers posse strong anti-oxidant and anti-inflammatory properties and maintain metabolic hemostasis.[12] In addition, the use of medicinal plants is believed to enhance brain function owing to modulatory effects on the gut-brain axis and neurochemical transmission.[13] Accumulating research have suggested that herbal supplements and fruits may be beneficial as alternative safe options for the management of anxiety and other psychiatric illness.[14]

Traditional Mediterranean fruits are an excellent source of therapeutic agents for a wide range of disorders, including psychiatric illnesses.[15] Dates, which are fruits of the date palm tree **(Phoenix dactylifera),** are a staple food in many Middle Eastern countries that are oval-shaped fruits, yellow in color, some of which tend to be red, with sweet taste.[16] Dates are among the fruits that Arabs and Muslims hold in high regard and consume on regular basis, particularly during the fasting month of Ramadan.[17] Dates have been reported to have various health benefits including anti-tumor, antioxidant and anti-inflammatory properties.[17] In addition, dates have been traditionally claimed for their ability to strengthen nerves and improve cognition and memory, resulting in the person becoming calm, clear-minded and alert.[18]

There are several types of dates, among which Medjool dates are traditionally known as "king of dates" owing to their rich texture, large size and wonderful taste, which combines the flavor of caramel, wild honey and some cinnamon. Medjool dates are originally from Morocco, but they have since moved to the United States, Iraq, Iran, South Asia, Jordan, and Palestine. Jordanian Medjool dates, which constitute 14% of global production, appear to achieve the rigorous standards for Medjool date farming. The Jordan Valley, which is the lowest area in the world above sea level, is considered one of the most suitable regions in the world for the production of Medjool dates due to its distinctive weather and fertile soil.[19] Though there have been many traditional claims on the therapeutic properties of Medjool dates, little is known about their neurobehavioral effects when consumed on a daily basis. According to previous studies, Medjool dates contain phytoactive compounds like flavonoids, phenolics and sterols which have been shown to affect brain functions.[20] In this regard, the current research was carried out to see if an ethanol extract of Medjool dates could have therapeutic benefits on the central nervous system. The present work aimed to investigate the possible anxiolytic-like effects of the ethanolic extract of Medjool dates in mice using the elevated zero-maze and marbles burying tests as animal models of anxiety. We hypothesized that supplementation with Medjool dates extract could reduce anxiety-like behaviors in mice.

MATERIALS AND METHODS

Chemicals

Ethanol (96%) was obtained from EMSURE (Darmstadt, Germany). Diazepam and Fluoxetine were

obtained from Sigma Aldrich (Massachusetts, USA).

Dates fruit extraction

One kilogram of fully ripe Tamr stage date palms (Medjool variety) was obtained from Haroun Al-bader Al-Odwan farm, in West Shona-Jordan. The harvest was of October 2020. Medjool dates *(Phoenix dactylifera L. cv. Medjool)* identified by Professor Nihad AlSmairat from the University of Jordan referred to The Illustrated Guide to the Cultivation of Date Palms and Dates book.[21]

***Phoenix dactylifera* Ethanolic Extract Preparation**

First, seeds were removed from the *Phoenix dactylifera* fruits. The flesh was cut into very small pieces and separated into many trays. As *Phoenix dactylifera* is very wet, the small pieces were covered using soft fabric, then dried under the sun for a week. After that, the fruits were dried using Gallenkamp oven (CAMBRIDGE, UK) for about three weeks at 50^{o}C until they were completely dry. The dried pieces were pulverized into fine particles using MEGA coffee grinder (China), mortar, and pestle, then sieved until we obtained a sugar-like texture. Finally, the powder was stored in a dry glass container.

Dry *Phoenix dactyilfera* powder was macerated in absolute ethanol, using the method of El Abed H *et al.* with minor modification.[22] Briefly, 15g of powder were macerated in 100ml absolute ethanol, and stirred for 24 hours at room temperature, using a hot plate (Thermo SCIENTIFIC CIMAREC) with magnetic stirrer (STUART SCIENTIFIC-UK). The extract was filtered using *Xinxing* Qualitative filter paper 125mm. Ethanol was evaporated and disposed using Rotavapor device associated with a heating bath R-300 (BUCHI Labortechnic, Switzerland) and vacuum pump V-700 (BUCHI Labortechnic, Switzerland). The extract became viscous and had acaramel-like texture and was kept in the round bottom flask for few hours for air drying, then it was stored in a dry glass container and was ready to be used. Each amount of the extract was dissolved in distilled water before use.

Phytochemical Screening of *Phoenix dactylifera* Ethanolic Extract

Phytochemical screening tests were used to detect the secondary plant metabolite groups including alkaloids, flavanoids, steroids, saponins, and tannins present in the ethanolic extract, using standard procedures with some modifications (Table 1).[23, 24]

Table 1. Phytochemical Screening Tests

Test	Procedure	Observation in case of positive result	Indication
Ferric Chloride test	Crude extract was mixed with 2ml of a 2% $FeCl_3$ solution	Blue-green or blue-black color	Polyphenols and Tannins
Frothing test	Crude extract was mixed with 5ml distilled water and vigorously shaken for 30 seconds.	Formation of stable and high froth, more than 1cm	Saponins
Cyanidine test	Two milliliter of concentrated hydrochloric acid was added to a half gram (0.5g) of crude extract dissolved in methanol. Effervescene was observed by adding a spatula full of magnesium turnings to the mixture.	Presence of brick reddish color.	Flavonoids
Dragndroff's test	In a test tube, the extract was mixed with 1ml of dragendroff's test.	Orange to reddish brown precipitate.	Alkaloids

Test	Procedure	Observation in case of positive result	Indication
Lieberman-Burchard test	About 0.5g of the crude extract was dissolved in 0.5ml dichloromethane to give a dilute solution and then 0.5 ml of acetic anhydride added, followed by three drops of concentrated sulphuric acid.	A blue-green coloration	Steroids

The exact phytochemicals compounds in the extract were detected by Liquid Chromatography with tandem mass spectrometry LC-MS/MS (SHIMADZU, **Japan).**

Husbandry of experimental animals

Bulb-c mice (weighing 20–25 g) were obtained from animal house of Al-Zaytoonah University of Jordan (Amman, Jordan). All mice were housed under regulated conditions (22-25°C, a 12 h light-dark cycle) in polyacrylic cages. The mice were cared in accordance with the guide of the Canadian Council on Animal Care,[25] and the study protocol was approved by the ethical committee at Al-Zaytoonah University of Jordan (2017–2016/22/40).

Animal groups and treatments

The mice were divided into four groups (six animals per cage), acclimatized to laboratory conditions for 7 days, and then treated as follows:

Negative control: mice of this group were given free access to drinking water without receiving any drugs or plant materials.

Positive control: mice of this group were treated with diazepam (1.5 mg/kg) or fluoxetine (5 mg/kg) at 30 minutes prior to behavioral experimentations. The duration of treatment was based on standardized protocol in the previous studies. [26-28]

Dates (low dose) group: mice were treated by 300mg/kg of *Phoenix dactylifera* extract dissolved in distilled water for 30 days prior to behavioral testing.

Dates (high dose) group: mice were treated by 2583mg/kg of *Phoenix dactylifera* extract dissolved in distilled water for 30 days prior to behavioral testing.

The low dose (300mg/kg) of *Phoenix dactylifera* extract was chosen because it has previously been found to be a functional and effective dose in a mouse study.[26] The high dose of *Phoenix dactylifera* extract (2583mg/kg) is similar to one piece of *Phoenix dactylifera* fruit consumed in one day by a human. One date weighs about 15g, and the equivalent dose was converted from human to mouse dose in mg/kg by multiplying the human dose by 12.3, the constant used to calculate the mouse dose based on the surface area difference between human and mouse.[29]

The observers of the behavioral tests were blinded to the treatments in all experimental sessions.

Behavioral tests

Elevated zero maze test

Anxiety behaviors were assessed using the elevated zero maze (EZM) apparatus. The anxiety behaviors were evaluated using a method described previously in the literature. [30, 31] The EZM is made up of four quadrants (two closed quadrants enclosed by 30 cm high walls, and the other two open quadrants left uncovered), that have a 5cm wide circular corridor, and are placed so that the two quadrants of each kind are opposite each other. The maze has a diameter of 60cm and stands 50cm off the ground. The test began with each mouse being placed in an open quadrant, with its head facing one of the closed arms. The latency to the closed arms, time spent in the closed arms, and the total number of enters to open quadrants were measured as indices for anxiety.

Marbles burying test

This experiment was conducted in a polypropylene mouse cage (42x24x12cm) with a metal grid top as described previously.[32] The floor of the cage was coated with a 5 cm

thick layer of bedding material, with 20 clean glass marbles (diameter 1.5 cm) were evenly arranged on top in a parallel pattern. The experiment was conducted by placing each mouse individually in the cage for 30 minutes. At the end of the experimental session, the mouse was removed, and the number of marbles buried to at least two-thirds was counted and used as an index of anxiety. Fluoxetine was used as a reference drug to assess the burying activity. Fluoxetine, the drug of choice for treatment of obsessive-compulsive disorder (OCD), has been frequently used as a reference in the marbles burying test. [27, 33]

Statistical Analysis

Results obtained from animal experimentation were presented as mean ± SD. Graph pad prism (www.graphpad.com) was used to conduct comparative measurement using One-way ANOVA with Tukey's post-test. A probability level (p-value) of 0.05 or less was considered significant.

RESULTS

Phytochemical Screening of *Phoenix dactylifera* ethanolic Extract

Phytochemical screening confirmed the presence of various phytochemical components, including polyphenols, tannins, flavonoids and steroids. A positive sign (+) indicates the presence of the compound while negative sign (-) indicates the absence of that compound (Table 2).

Table 2. Qualitative phytochemical screening of *Phoenix dactylifera* extract

Compound	Result
Polyphenols and Tannins	+
Saponins	_
Flavonoids	+
Alkaloids	_
Steroids	+

The following bioactive phytochemical compounds were identified using LC-MS/MS: kaempferol, campesterol, lutein, apigenin, genistein, isoquercetin, isofucosterol, neoxanthin, stigmasterol, β-carotene, glycitein, violaxanthin, antheraxanthin, formononetin, caffeine, β-sitosterol, quercetin, diadzein, rutin, secoisolariciresinol, ferulic acid, pelargonin, coumestrol, syringic acid, sinapic acid, luteolin, pinoresinol, chlorogenic acid, lariciresinol, o-cinnamic acid, caffeic acid, catechin, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, matairesinol, gallic acid, and resveratrol.

Effect on anxiety behaviors in EZM **test**

Data obtained from the EZM test are shown in Fig.1-3. The results showed that chronic intake of extract at low concentration produced considerable anxiolytic effects, determined by a significant increase in the latency to the closed area, a significant decrease in time spent in the closed area and a significant increase in the total number of entries to the open areas as compared to the effect of the negative control group. These effects were comparable to those produced by diazepam. On the other hand, chronic intake of high concentration did not reduce anxiety-like behaviors at all tested parameters.

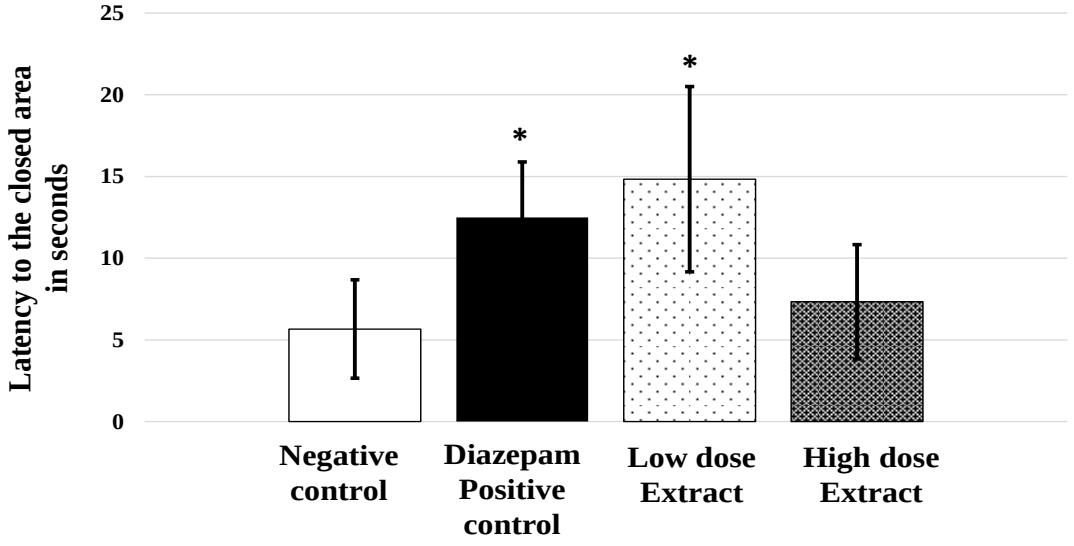

Figure 1: Latency to the closed arms. (*) indicates significant difference from negative control group. One-way ANOVA results (df=3, F=6.8, p value= 0.002). Post-hoc Tukey HSD test results showed significant difference between group received diazepam and negative control (p value=0.0037) and significant difference between group received low dose of extract and negative control (p value=0.004).

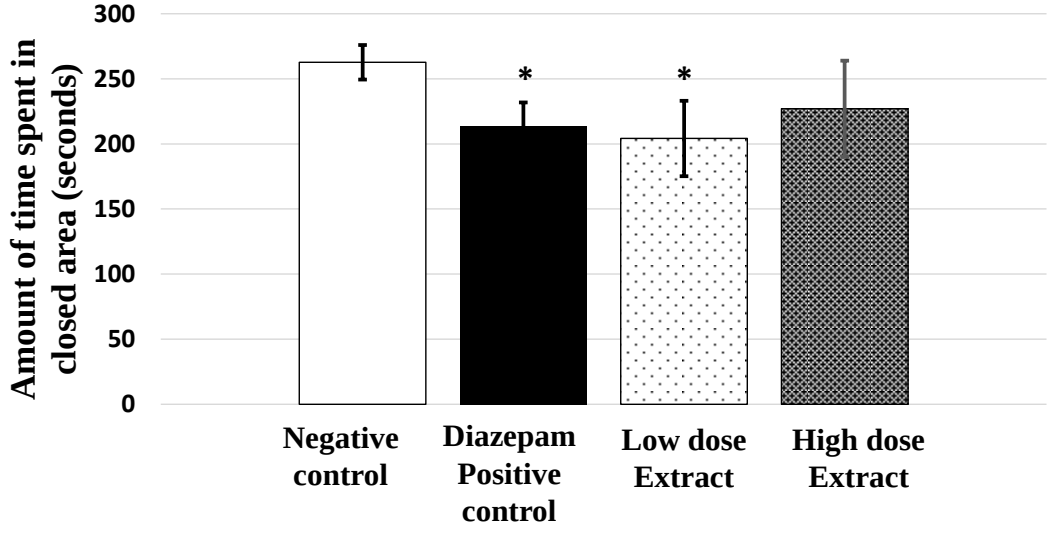

Figure 2: Amount of time spent in the closed arms.

(*) indicates significant difference from negative control group. One-way ANOVA results (df=3, F=5.8, p value= 0.005). Post-hoc Tukey HSD test results showed significant difference between group received diazepam and negative control (p value=0.02) and significant difference between group received low dose of extract and negative control (p value=0.004).

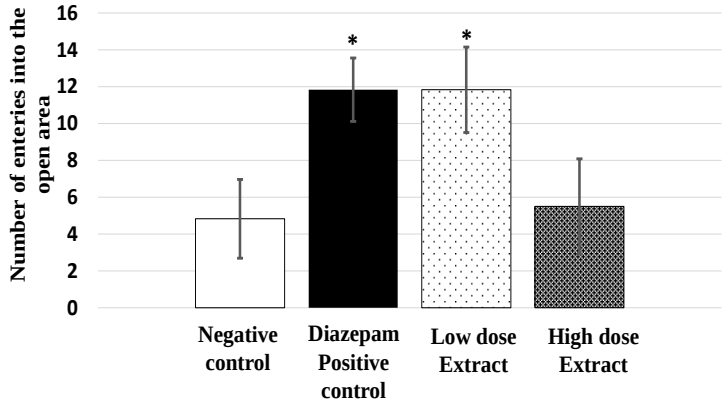

Figure 3: Number of entries into open area.

(*) indicates significant difference from negative control group. One-way ANOVA results (df=3, F=18.2, p value= 6.1e-0.6). Post-hoc Tukey HSD test results showed significant difference between group received diazepam and negative control (p value=0.001) and significant difference between group received low dose of extract and negative control (p value=0.001).

4.2. Effect on marbles burying behavior

The findings of the marbles burying test are presented in Fig.4. The results showed that mice subjected to chronic intake of low concentration of the extract showed significant decrease in marbles burying behavior as compared to the counterparts of the negative control. The reduced burying behavior by these mice was comparable to that observed in the fluoxetine treating group. In contrast, mice exposed to the high concentration of the extract did not show any significant decrease in the marbles burying behavior as compared to mice of negative control.

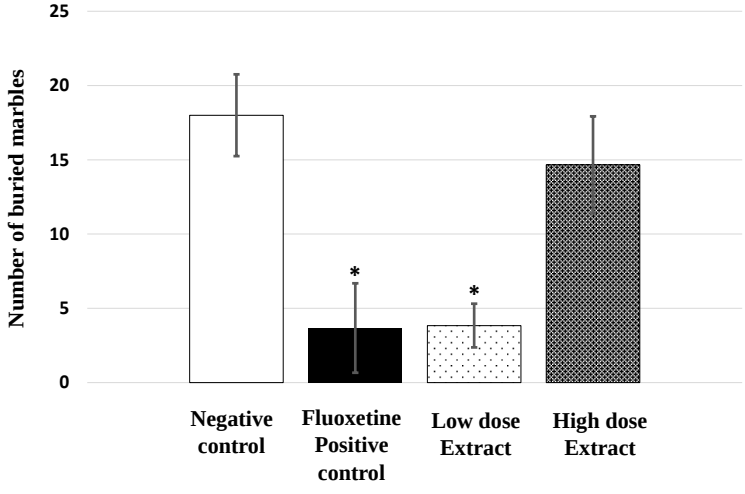

Figure 4: Number of marbles buried during marbles burying test.

(*) indicates significant difference ($p<0.05$) from negative control group. One-way ANOVA results (df=3, F=44.5, p value= 4.9e-09). Post-hoc Tukey HSD Test results showed significant difference between group received fluoxetine and negative control (p value=0.001) and significant difference between group received low dose of extract and negative control (p value=0.001).

DISCUSSION

The cultivation of the Medjool date palm in Jordan is a relatively recent story that has achieved widespread economic success in recent decades.[19] The Jordan Valley, the lowest region in the world above sea level, is known for its great agricultural potential, which is characterized by exceptional climatic conditions including hot summer and moderate winter, as well as the abundance of water supply and fertile soil.[34] Accordingly, there is a strong popular belief that Medjool dates grown in the Jordan Valley may exert a great beneficial effect for the maintenance of body homeostasis that has not yet been verified. In this regard, the present work was conducted to prepare an ethanolic extract of Medjool dates from Jordan Valley and evaluate its behavioral effects in mice. The chemical analysis revealed that the whole ethanolic extract of Medjool dates contains phytochemical secondary metabolite groups, such as phytosteroids and flavonoids, that had been previously identified to be effective as anxiolytic agents in prior studies (Table 2). These chemicals are supposed to work as a combination therapy for the management of anxiety by acting on several molecular targets (Table 3). In many cases, the use of whole plant extracts provides more pharmacological benefits than using single separated components.[35] Aside from their potential to concentrate the bioactive chemicals, the co-existence of phytochemicals in the whole extracts may exert an interplay effect in terms of enhancing the overall pharmacokinetic and pharmacodynamic effects.[36] In various toxicological research in animals, the use of the whole date extracts from different solvents was shown to exhibit a neuroprotective effect against oxidative damage, neuronal damage, spatial learning and memory impairment.[37, 38] However, this study was conducted to support the conventional claim that regular consumption of dates improves brain function and maintains nervous system homeostasis, including the potential to induce fearlessness trait. In behavioral research, the use of laboratory models with a specific design, known as "mazes", is a typical method.[14, 31] The term "maze" is used in behavioral studies to express a complex design of interlocking passages that is shaped in a way that stimulates distinctive behavioral responses. [39] Within maze apparatus, laboratory animals display a set of well-recognized behaviors that can be quantitively analyzed to assess the emotional and cognitive functions.[40] The zero-maze apparatus is one of the most sensitive and reliable paradigms for evaluating anxiety-like behavioral in mice. [41, 42] From an experimental point of view, the EZM test provides relatively stable results for multiple trials throughout several days and weeks.[42] The functional principle of the EZM test is based on the fact that mice display natural aversion toward the open area while preferring to hide themselves in a closed area from potential threats. In particular, the anxiety behaviors in the EZM test were found to be highly sensitive to the drugs that act selectively on the GABAergic nervous system such as diazepam.[41] In this study, data obtained from the EZM test showed that supplementation with low concentration of the date extract caused a significant anxiolytic effect that was comparable to the diazepam effects. Collectively, these results suggest that Medjool Dates will be helpful for the prevention and management of anxiety disorders, which may be attributed in part to a modulatory effect on the GABAergic system. The GABAergic effect could be partially related to the presence of some GABA modulators in the extract such as kaempferol and stigmasterol.[43]

The anxiolytic effect of the ethanolic extract was also evidenced in the marbles burying test. Marble burying behavior has been examined frequently in research to assess anxiety-related compulsive behavior.[44] In Psychiatry Diagnostic & Statistical Manual of Mental Disorders (3rd Edition, 3rd Edition Revised, 4th and 5th editions), obsessive-compulsive disorder (OCD) was classified as an anxiety disorder.[45] It has been well recognized that anxiety behavior in the marbles burying test has a compulsive component, indicating that mice fail

to habituate to anxiety even after several trials of the experiment.[32] As a result, this test is commonly regarded as a reliable paradigm for studying obsessive-compulsive disorders.[46] The principle of this experiment is based on the frequent tendency of mice to bury marbles as frightening objects.[44] It is thought that burying behavior is controlled by the serotoninergic system.[44] Fluoxetine, a serotonin reuptake inhibitor, has been frequently used as a reference in the marbles burying test. Accordingly, the reduced burying behavior by ethanolic extract of the dates may partially indicate the involvement of the serotoninergic system in the mechanism of action. Moreover, the presence of various neuromodulators that act on the serotonergic nervous system, such as genistein and ferulic acid, may contribute to the serotoninergic effect.[47, 48]

In both the EZM and marbles burying tests, high dose consumption of the extract was ineffective in reducing the anxiety behaviors in mice. This could be due to saturated pharmacokinetics, suggesting significant interaction between various components present in the extract.[49, 50] In addition, the concentrated ethanolic extract may contain a sufficient amount of anxiogenic chemicals that may counteract the anxiolytic effect of other components present in the extract. For example, caffeine, which was found in trace amounts in Medjool dates, may present at a relatively high dose in the concentrated extract causing a paradoxical anxiogenic effect in mice.[51, 52]

Table 3. Examples of anxiolytic chemicals available in the Medjool date extract

Chemicals	Examples	Effect on animal model (s)	References
Phytoestrogens	Genistein	Relieving anxiety-like behaviours in ovariectomized rats and in post-traumatic stress disorder models	35
		Regulating symptoms of obsessive-compulsive disorders in streptozotocin-induced diabetic mice	40
Phytosterols	Stigmasterol	Exerting anxiolytic and anticonvulsant effects in pentylenetetrazol-treated mice	43
	B-sitosterol	Alleviating anxiety and exerting a synergetic anxiolytic effect with established anxiolytic drug in mice	53
Flavonoids/ isoflavones	Kaempferol	Exerting anxiolytic activities in the elevated plus maze test in mice	54
	Formononetin	Protecting neurons from N-Methyl-D-aspartate-evoked excitotoxic injury and exerting anxiolytic effect in an inflammatory pain mouse model	**55**
	Quercetin	Mitigating anxiety-like behavior and normalizing hypothalamus-pituitary -adrenal axis hypersensitivity in a mouse model of mild traumatic brain injury	56
	Rutin	Reducing anxiety-like behaviors in pentylenetetrazol-treated mice	57
Xanthians	Caffeine	Producing anxiogenic and anxiolytic effects at dose dependent manner	52
Phenolic acids/ benzoic acid	Ferulic acid	Alleviating anxiety and other abnormal behaviours in isolation-reared mice and promoting anxiolytic effect in the light-dark test in zebrafish	**48**

Chemicals	Examples	Effect on animal model (s)	References
	Caffeic acid	Producing anxiolytic effect in elevated plus maze test in mice	**58**
	Vanillic acid	Reducing anxiety like behavior and impulsivity following global cerebral ischemia in rodents	59
	Gallic acid	Inducing anxiolytic effect in streptozotocin-induced diabetic mice	60

Table 4. underlying mechanism of anxiolytic effects of various chemicals in the ethanolic extract

Chemicals	Mechanism of action	References
Genistein	Enhancing serotonergic transmission in the amygdala	40
Stigmasterol	Exerting a modulatory effect on Gamma-aminobutyric acid system, particularly by enhancing GABA-induced currents at $GABA_A$ receptors	43
Quercetin	Normalizing hypothalamus-pituitary -adrenal axis hypersensitivity	56
Ferulic acid	5-HTA receptor partial agonist activity	48
Caffeic acid	N-methyl-D-aspartate receptor antagonist activity	61, 62, 63

CONCLUSION

Results from the current work suggest that ethanolic extract of Medjool dates may contain bioactive compounds that are capable of reducing anxiety and compulsive behaviours. The underlying mechanism of anxiolytic effect may involve several molecular targets including, at least in part, a modulatory effect on the GABAergic and serotoninergic nervous systems. Overall, this study demonstrated that ethanolic extract of Medjool dates could be explored as a valuable source of phytoactive compounds that may provide safe and effective treatment options for a variety of anxiety disorders. However, more researches are needed to map out the total anxiolytic effects of these dates using extract from other solvents.

One limitation of the present study is that only two doses were used, with a considerable gap between them, making it difficult to determine whether the anxiolytic effect of dates extract was dose-dependent. In addition, further investigations are needed to confirm the anxiolytic effect of Medjool dates using additional behavioral models such as the open field, light dark box, and acute restrain tests.

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Declaration of Interest

The authors report that they have no conflicts of interest.

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التأثير المزيل للقلق للمستخلص الكحولي الإثانولي لتمر المجهول على الفئران المخبرية

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ملخص

الخلفية: القلق هو أحد الاضطرابات النفسية الأكثر شيوعًا، حيث يصيب 33.7٪ من عامة السكان. ومع ذلك، فقد وجد أن استهلاك الأنظمة الغذائية الصحية يساعد، على الأقل جزئيًا، في الوقاية من الاضطرابات الشبيهة بالقلق وعلاجها .

الطرق البحثية: في هذه الدراسة، تم تقييم سلوكيات القلق لدى الفئران التي تعرضت للتناول المزمن لجرعات منخفضة وعالية من المستخلص الكحولي لتمور المجهول (Phoenix Dactylifera) مقارنة بنظيراتها من الفئران الضابطة التي لم تتناول التمر. تم استخدام اختبار ارتفاع متاهة الصفر واختبار دفن كرات الرخام كنماذج مفضلة لتقييم سلوكيات القلق لدى هذه الفئران. بالإضافة إلى ذلك، تم إجراء تحليل كيميائي نباتي لمجموعات الأيض الثانوية الرئيسية لمستخلص تمر المجهول .

النتائج: بينت نتائج هذه الدراسة أن مستخلص تمر المحهول غني بالفلافونويد والستيرويدات مع نشاط معروف باسم مزيلات القلق مثل الكايمفيرول. أظهرت الفئران التي تلقت جرعة منخفضة (300 مجم / كجم) من المستخلص قلقًا أقل من خلال استخدام اختبار ارتفاع متاهة الصفر من الفئران التي لم تتناول مستخلص التمر، والذي تم تحديده من خلال زيادة كبيرة في زمن الوصول إلى المنطقة المغلقة و انخفاض كبير في الوقت الذي يقضيه في المنطقة المغلقة و أيضا زيادة كبيرة في عدد الإدخالات إلى الأرباع المفتوحة. كان تأثير مزيل القلق لمستخلص التمر من الجرعة المنخفضة مشابهًا للتأثير الناتج في الفئران التي عولجت بالديازيبام (1.5 مجم / كجم) في جميع المتغيرات المختبرة الدالة على مستوى القلق. باللإضافة إلى ما سبق، أظهرت نتائج اختبار دفن كرات الرخام فرقا احصائيا في إزالة القلق بجرعة منخفضة (300 مجم / كجم) من مستخلص التمر مقارنة بالفئران التي لم تتناول مستخلص التمر، والذي تبيين من خلال إنخفاض في العدد الإجمالي لكرات الرخام المدفون. إضافة إلى ذلك، كان التأثير المزيل للقلق لمستخلص التمر من الجرعة المنخفضة في اختبار دفن كرات الرخام مشابهًا للتأثير الناتج في الفئران التي عولجت بفلوكستين (5 مجم / كجم). من ناحية أخرى، فإن تناول جرعات عالية من مستخلص التمر (2583 مجم / كجم) لم يسبب أي تأثير مزيل للقلق في متاهة الصفر المرتفعة واختبارات دفن كرات الرخام.

الاستنتاجات: بشكل عام، تشيرالنتائج هذه الدراسة إلى أن تناول جرعة منخفضة بانتظام من المستخلص الكحولي من التمر المجهول قد يساعد في منع وإدارة اضطرابات القلق. ومع ذلك، يوصى بإجراء مزيد من الدراسات لتوضيح الآلية المفترضة الكامنة وراء التأثير المزيل للقلق لدى تمر المجهول.

الكلمات الدالة: القلق، متاهة الصفر المرتفعة، فينيكس داكتيليفيرا، دفن كرات الرخام، تمر المجهول.

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Evaluation of the Antimicrobial Activity of *Strombosia grandifolia* Hook.f. ex Benth Extract Hand Sanitizer Formulation

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ABSTRACT

The hand is an easy agent for the spread and transmission of pathogens and community-acquired infections. This study aims at evaluating the antimicrobial activity of *Strombosia grandifolia* hand sanitizer compared with a commercially available alcohol-based hand sanitizer. Water, methanol, and ethyl acetate extract from the leaf of *Strombosia grandifolia* was used to formulate hand sanitizer. The antimicrobial activity of the hand sanitizers was carried out against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis and Candida albicans* and analyzed with one-way ANOVA. The sanitizers were smooth with a chilly feel on the skin. Aqueous and methanol extract sanitizer had a significantly better antimicrobial activity when compared with ethyl acetate extract with the rank order of susceptibility of the microorganisms as *Staphylococcus aureus* > *Bacillus subtilis* > *Pseudomonas aeruginosa* > *Escherichia coli* > *Candida albicans*. The incorporation of the extract synergized the antimicrobial activity of alcohol-based hand sanitizers.

Keywords: *Strombosia grandifolia*, antimicrobial activity, phytochemical screening, hand sanitizer.

INTRODUCTION

Hygiene is a condition and practice that helps to maintain health and prevent the spread of disease. Cleanliness is a necessity for the maintenance of well-being, so good hygiene and the usage of cleansers are requisite for healthy living. The hand is an easy agent for the spread and transmission of pathogens and community-acquired infections.[1] Proper hand hygiene can avert health care associated infections and reduce the spread of antimicrobial resistance.

Hence, hand hygiene is a term that refers to any action of hand cleansing which is an essential precautionary method to prevent the transmission and spread of infections.[2] To achieve this, soap and water had been traditionally used to reduce microbial load.[3] The use of soap and water is less effective in killing microorganisms and it is not very convenient and time-wasting in situations where health personnel will have to attend to so many clients,[4,5] and in the absence of water, it is practically impossible to use soap and water to cleanse the hand.

Poor hand hygiene practices thrived in West Africa until the outbreak of the Ebola virus due to a lack of awareness, lack of knowledge of risk and non-availability of hand hygiene facilities.[6] The Ebola virus disease outbreak in 2014 - 2016 was the genesis of the emphasis on the use of hand sanitizers in the West African sub-region as an infection control measure. This has made researchers to continue in the search for discovering and developing new hand sanitizers and improving on existing

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ones. Nwabueze et al.[7] reported hand washing and use of hand sanitizers as measures to prevent Ebola virus disease. World health organization also advocated the use of hand sanitizers globally as one of the measures to prevent the spread of infectious diseases such as the recent coronavirus disease pandemic.[8] Hand rubs have the advantage of good antimicrobial activity in a short time unlike the use of water and soap.

Strombosia grandifolia (*S. grandifolia*) Hook.f. ex Benth is a tree which grows up to 30m high and belongs to the family Olacaceae. It has simple and broad leaves. It is distributed mostly in lowland forests in tropical Africa. The plant is known in Yoruba, Nigeria as "Itako pupa" and it is used as an ethnomedicinal herb in the treatment of various infectious diseases such as skin infections, gonorrhea, cough and cold possibly because of the presence of antimicrobial phytochemicals in the plant. [9] Very little report on *S. grandifolia* is available in literature. Ekalu et al.[9] reported antibacterial and antifungal activities of the stem bark of the plant. This study attempts incorporating various extracts of the leaf of *S. grandifolia* into an alcohol based hand sanitizer with the aim of improving its antimicrobial activities.

MATERIALS AND METHODS

Materials

The fresh leaves of *S. grandifolia* were collected from Sapoda, a village in Edo state, Nigeria in the month of June 2018. The plant was identified and authenticated by Mr A. S. Odewo of the Forestry Research Institute of Nigeria, Ibadan, Oyo state (Voucher Number: FHI 111932). The chemicals and reagent used were analytical grades purchased from BHD chemicals, Poole England

Plant Extraction

The leaves were washed and air dried for 7 days after which it was milled into powder with the aid of a mechanical blender. Two hundred gram (200g) of the powdered leaves was macerated in 500mL of water, methanol, and ethyl acetate for 72 hours at room temperature with intermittent shaking. The mixture was filtered and the filtrate was concentrated using a rotary evaporator and dried.

Phytochemical Analysis

Qualitative phytochemical screening of the extracts was done by standard methods according to Trease & Evans.[10]

Test for Alkaloids

To 1g of the powdered leaf, 10mL of 10% HCl was added and heated on water bath for 10 min. The mixture was filtered and 1 mL of the filtrate was transferred into four test tubes. Few drops of Dragendorff's reagent, Mayer's reagent, Wagner's reagent and 1% tannic reagent were added to test tube 1, 2, 3 and 4 respectively. Formation of an orange, light brown, reddish brown and golden yellow precipitates in test tube 1, 2, 3 and 4 respectively indicate the presence of alkaloids.

Test for Tannins

One gram (1g) of the powdered leaf was boiled for 5 min. in 20mL of water. It was filtered after cooling. One milliliter (1mL) of the filtrate was mixed with 5mL of water in a test tube and few drops of 0.1% ferric chloride solution were added. A greenish colour indicates the presence of tannins.

Test for Saponins (Frothing test)

One gram (1g) of the powdered leaf was boiled for 10 min. in 10mL of water. The mixture was filtered while hot and the filtrate was allowed to cool. Three milliliters (3mL) of the filtrate was mixed with 10ml of water and shaken vigorously for 2 min. The formation of froth on the upper surfaces of the liquid indicates the presence of saponin.

Test for Cardiac Glycosides

One gram (1g) of the powdered leaf was extracted with 10mL of 80% alcohol for 5 minutes on a water bath. The filtrate was diluted with equal volume of water. Two milliliters (2mL) of lead acetate was added and the mixture was filtered after standing for few minutes. Two milliliters (2mL) of concentrated H_2SO_4 was added along the side of the test tube to the filtrate. The formation of a light reddish brown colour at the interface with a green colour in the

acetic layer indicates the presence of cardiac glycosides.

Test for Anthraquinone

One gram (1g) of the powdered leaf was placed in a dry test tube and 10mL of chloroform was added and shaken for 5 min. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. The formation of a bright pink colour in the aqueous layer indicates the presence of free anthraquinones.

Test for flavonoids

One gram (1g) of the powdered leaf was boiled with 10mls of water for 5 min. and filtered while hot. The filtrate was allowed to cool. Two milliliter (2mL) of NaOH was added to 5mls of the cooled filtrate. A yellow colour indicates the presence of flavonoid.

Five milliliter (5mL) of dilute ammonia solution was added to 10mL of the cooled filtrate followed by the addition of 3mL of conc. H_2SO_4. Colour disappearance on standing indicates the presence of flavonoid.

Antimicrobial Evaluation of Plant Extracts

The screening of antimicrobial activity of the plant extracts was carried out against five test microorganisms (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* 27853, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6051 *and Candida albicans* **ATCC 10231**) using the agar well diffusion technique according to the method described by Adeleye et al.[11] A standardized inoculum of each test microorganism was inoculated into sterile, cooled Mueller Hinton or Sabouraud dextrose agar, thoroughly mixed and transferred into a sterile Petri dish. After the agar had set, three holes were bored using a 6mm cork borer. The holes were filled with 0.5mL of 100mg/mL plant extract reconstituted with distilled water (DS), 0.5mL of 1% ciprofloxacin (positive control for bacterial) or 0.5mL of 1% fluconazole (positive control for fungal) and DS (negative control) respectively. The plates were allowed to stand for about one hour before incubation at 37^{o}C for 24 hours for Mueller Hinton plate and at 25^{o}C for 72 hours for Sabouraud dextrose agar. The zones of growth inhibition were then measured to the nearest millimetre.

Formulation of *S. grandifolia* Hand Sanitizer

The herbal hand sanitizer was prepared containing various concentrations of *S. grandifolia* extract as shown in Table 1. A modification of the method described by WHO, was adapted in the formulation of the herbal hand sanitizer. [12] The required quantities of the ingredients (Table 1) were accurately measured into a flask which was then shaken gently to mix the contents.

Table 1: Composition of herbal hand sanitizer formulations

MATERIALS	NO	A1	A2	A3	A4	A5	M1	M2	M3	M4	M5	E1	E2	E3	E4	E5
Isopropyl alcohol ($\%^{V}/_{V}$)	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70
S. grandifolia extracts ($\%^{w}/_{V}$)	-	0.5	1.0	3.0	5.0	10	0.5	1.0	3.0	5.0	10	0.5	1.0	3.0	5.0	10
Glycerol ($\%^{V}/_{V}$)	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Distilled water	26	25.5	25	23	21	16	25.5	25	23	21	16	25.5	25	23	21	16

NO- Hand sanitizer without plant extract, A1- 0.5% *S. grandifolia* aqueous extract, A2- 1.0% *S. grandifolia* aqueous extract, A3- 3.0% *S. grandifolia* aqueous extract, A4- 5.0% *S. grandifolia* aqueous extract, A5- 10% *S. grandifolia* aqueous extract, M1- 0.5% *S. grandifolia* methanol extract, M2- 1.0% *S. grandifolia* methanol extract, M3- 3.0% *S. grandifolia* methanol extract, M4- 5.0% *S. grandifolia* methanol extract, M5- 10% *S. grandifolia* methanol extract, E1- 0.5% *S. grandifolia* ethyl acetate extract, E2- 1.0% *S. grandifolia* ethyl acetate extract, E3- 3.0% *S. grandifolia* ethyl acetate extract, E4- 5.0% *S. grandifolia* ethyl acetate extract, E5- 10% *S. grandifolia* ethyl acetate extract.

Physical characterization of the Herbal Hand Sanitizer

Physical evaluation of the hand sanitizer was done manually by observing colour, odour, texture and feel on the skin.[13,14] The pH of the formulations was determined with a pH meter.

Antimicrobial Evaluation of Formulated Hand Sanitizers

The method used for the screening of antimicrobial activity of the plant extracts against the five test microorganisms was utilized for the formulated hand sanitizers.

Statistical analysis

Microsoft Excel 2010 and GraphPad Prism 5 were used to analyze data obtained. Data were presented as mean ± standard derivation (SD). One-way analysis of variance and Tukey's Post Hoc test were used to check significant differences in mean. Parameters with p-value of < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical test

The result of the phytochemical screening of the crude extracts is presented in Table 2. The powdered leaves contain moderate concentrations of alkaloids, saponins, flavonoids and tannins. Aqueous extract contains moderate concentrations of alkaloids with low concentration of saponins, flavonoids and tannins. Methanol extract contains moderate concentrations of alkaloids, saponins and tannins with low concentration of flavonoids, while ethyl acetate extract contains low concentrations of alkaloids, saponins and flavonoids.

Plants possess some secondary metabolites that protect them from attack by microorganisms and insects such as alkaloids, flavonoids, tannins, phenols, saponins, and other aromatic compounds.[15] The phytochemical screening (Table 2) indicated that the plant extracts generally contain alkaloids, saponins, flavonoids and tannins. Plants synthesize flavonoids in response to microbial invasion.[16] saponin acts by causing leakage of proteins and enzymes from the invading microorganism,[17] while tannins act by interfering with protein synthesis in the invading microorganism.[18] Since the different extracts of *S. grandifolia* leaves possess these secondary metabolites, there is the possibility of the plant being bioactive as an antimicrobial agent.

Table 2: Phytochemical Screening of *S. grandifolia* leaves

Secondary metabolites	Powdered leaf	Distilled water	Methanol	Ethyl acetate
Alkaloids	++	++	++	+
Cardiac Glycosides	-	-	-	-
Antraquinone	-	-	-	-
Saponins	++	+	++	+
Flavonoids	++	+	+	+
Tannins	++	+	++	-

- = Not detected; + = Low concentration; ++ = Moderate concentration;

Antimicrobial Activity of Plant Extracts

The result of the antimicrobial activity of the plant extract (100mg/mL) is presented in Table 3. All the extracts showed a significant difference in antibacterial and antifungal activity against all the test microorganisms with methanol extract having the highest antimicrobial activity. The rank order of antimicrobial activity was methanol > distilled water > ethyl acetate. The high antimicrobial activity of methanol extract as revealed in Table 3 could be due to the presence of high concentrations

of saponin and tannin than the other two extracts. Plants with extracts rich in tannin have been reported to show high antimicrobial activities mainly by hydrophobic interactions and hydrogen bonds leading to inhibition of bacteria metabolism.[19] Methanol is known to be a good solvent for extraction, extracting various chemical groups from plant materials in large quantities as reported by Adeleye et al.[11], Murugan et al,[19] Alayo et al. [20]

The aqueous and methanol extracts had strong antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. The rank order of susceptibility of the organisms to aqueous and methanol extracts was *Staphylococcus aureus* > *Bacillus subtilis* > *Pseudomonas aeruginosa* > *Escherichia coli*. Ethyl acetate extract had the lowest antibacterial activity against all the test microorganisms. Only methanol extract showed a significantly comparable antibacterial activity to that of the positive control, ciprofloxacin on *Bacillus subtilis* and *Pseudomonas aeruginosa* (Table 3) probably due to the reason earlier mentioned. The rank order of antifungal activity of the plant extracts was methanol > distilled water > ethyl acetate which is similar to the antibacterial activity. The positive antifungal control (fluconazole) had better activity than all the plant extracts.

Table 3: Antimicrobial activity of extracts of *S. grandifolia*

Test organisms	Zones of inhibition (mm)				
	A	M	E	B	F
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Staphylococcus aureus	12±0.01	16±0.39	04±0.10	20±0.04	-
Escherichia coli	08±0.11	11±0.24	06±0.08	16±0.01	-
Bacillus subtilis	10±0.04	16±0.08	07±0.24	18±0.06	-
Pseudomonas aeruginosa	12±0.08	14±0.11	08±0.02	16±0.10	-
Candida albicans	07±0.14	10±0.30	05±0.11	-	17±0.21

A = Aqueous extract, M = Methanol extract, E = Ethyl acetate extract, B = 1% Ciprofloxacin for bacterial positive control, F = 1% Fluconazole for fungi positive control,

Physical characteristics of the hand sanitizer formulations and pH

The physical properties of the hand sanitizer formulations and the pH of the formulations is presented in Table 4. The formulations exhibited different shades of green or brown coloration and they are all smooth in texture with a chilly feel on the skin. The pH of the formulations ranged from 6.0 to 7.5.

The ideal pH of the human skin is usually slightly acidic within the range of 4 – 6.5.[21,22] Formulations for application on the skin should be within this range to prevent skin irritation. As shown in Table 4, only four formulations (A4, M3, M4 and M5) had a pH value within the ideal range. The pH of other formulations can be made to be within the ideal range with the use of suitable buffer.

Table 4: Physical properties and pH of formulations

Formulation code	Colour	Texture	Feel on skin	pH
NO	Colour less	Smooth	Chilly	7.4
A1	Light Brown	Smooth	Chilly	7.1

Formulation code	Colour	Texture	Feel on skin	pH
A2	Light Brown	Smooth	Chilly	7.0
A3	Light Brown	Smooth	Chilly	7.2
A4	Light Brown	Smooth	Chilly	6.4
A5	Light Brown	Smooth	Chilly	7.0
M1	Light Brown	Smooth	Chilly	7.5
M2	Light Brown	Smooth	Chilly	7.2
M3	Brown	Smooth	Chilly	6.5
M4	Brown	Smooth	Chilly	6.3
M5	Brown	Smooth	Chilly	6.0
E1	Light Green	Smooth	Chilly	7.4
E2	Light Green	Smooth	Chilly	7.5
E3	Light Green	Smooth	Chilly	7.3
E4	Green	Smooth	Chilly	7.1
E5	Green	Smooth	Chilly	7.1
C	Light Blue	Smooth	Chilly	6.4

NO = formulation with no extract, A1-A5 = *S. grandifolia* aqueous extracts, M1-M5 = *S. grandifolia* methanol extracts, E1-E5 = *S. grandifolia* ethyl acetate extracts, C = commercially available hand sanitizer containing 45% Ethyl alcohol.

Antimicrobial Activity of herbal hand sanitizers

The antimicrobial activity of the leaves extract of *S. grandifolia* formulated into hand sanitizer was determined by the diameter (measured in mm) of the zone of inhibition and presented in Fig. 1, 2 and 3. The antimicrobial activities are concentration-dependent. Statistical analysis of the formulations containing 0.5% extracts of different solvents [A1, M1 & E1] showed no significant difference in activity against all the test microorganisms except *Bacillus subtilis* [P = 0.0270] with methanol extract. At 1% extract concentration [A2, M2 & E2] there was no significant difference in activity against *Staphylococcus aureus* and *Candida albicans* but there was a significant difference in activity against *Escherichia coli* [P = 0.0025] (the exact difference was between formulation A2 and E2; M2 and E2) with A2 and M2 having higher activity, *Pseudomonas aeruginosa* [P = 0.0080] (the exact difference was between formulation M2 and E2) with M2 having higher activity and *Bacillus subtilis* [P = 0.0025] with the exact difference between formulation A2 and M2; M2 and E2 with A2 and M2 having higher activity. At 3% extract concentration [A3, M3 and E3] there was significant difference in activity against all test microorganisms, *Staphylococcus aureus* [P = 0.0025] (the exact difference was between formulation A3 and M3; M3 and E3) with M3 having the highest activity, *Escherichia coli* [P = 0.0020] (the exact difference was between formulation A3 and M3; M3 and E3) with M3 having the highest activity, *Pseudomonas aeruginosa* [P = 0.0270] (the exact difference was between formulation M3 and E3) with M3 having higher activity, *Bacillus subtilis* [P = 0.0270] (the exact difference was between formulation M3 and E3) with M3 having higher activity and *Candida albicans* [P = 0.0066] with the exact difference between formulation A3 and M3; A3 and E3 with A3 having the highest activity. At 5% extract concentration [A4, M4 and E4] there was no significant difference in activity against *Pseudomonas aeruginosa* and *Candida albicans* but there was significant difference in activity against *Staphylococcus aureus* [P = 0.0025] (the exact difference

was between formulation A4 and E4; M4 and E4) with A4 and M4 having the higher activity, *Escherichia coli* [P = 0.0066] (the exact difference was between formulation A4 and M4; A4 and E4) with M4 having the highest activity and *Bacillus subtilis* [P = 0.0270] with the exact difference between formulation M4 and E4 with M4 having the highest activity. At 10% extract concentration [A5, M5 and E5] there was no significant difference in activity against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* but there was significant difference in activity against *Escherichia coli* [P = 0.0027] (the exact difference was between formulation M5 and E5) with M5 having the higher activity and *Candida albicans* [P = 0.0080] with the exact difference between formulation A5 and E5 with A5 having the higher activity. There was significant difference in the activity of all the formulations against the test microorganisms when compared with the commercially available hand sanitizer.

Statistical analysis of the results of the antimicrobial activity of herbal hand sanitizers as shown in shown Fig. 1, 2 and 3 revealed that aqueous and methanol extract of *S. grandifolia* hand sanitizer formulation had a significantly better antimicrobial activity when compared with ethyl acetate extract. However, methanol extract had a slightly better activity when compared with the aqueous extract although generally not statistically significant except the formulations containing 1% concentration against *Bacillus subtilis*, 3% concentration against *Staphylococcus aureus* and *Escherichia coli*, and 5% concentration on *Escherichia coli*. The reason for these variations in antimicrobial activities could be correlated to the concentration of the phytochemicals present in the extracts (Table 2). As highlighted previously, the presence of phytochemicals such as tannins, saponins and flavonoids could be responsible for these activities.[11,20,23] The extent of these activities may be due to the concentrations of these phytochemicals.[11] In this study, the probable mechanism of action of the plant extract as a result of the phytochemicals could be disruption of bacterial cell membrane as reported by Gonelimali et al.[25] The antimicrobial activity of the formulations were significantly higher against all the test microorganisms when compared with the commercially available hand sanitizer due to synergism as a result of the incorporation of the leaf extract of *S. grandifolia.* This synergistic effect may prevent microorganisms from developing alcohols-tolerance as highlighted by Golin et al. [26]

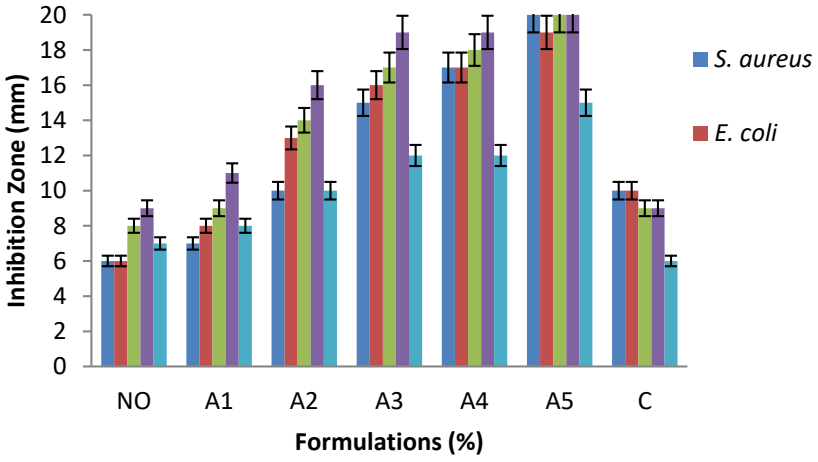

Figure 1: Zone of inhibition (mm) of aqueous extract hand sanitizer formulations

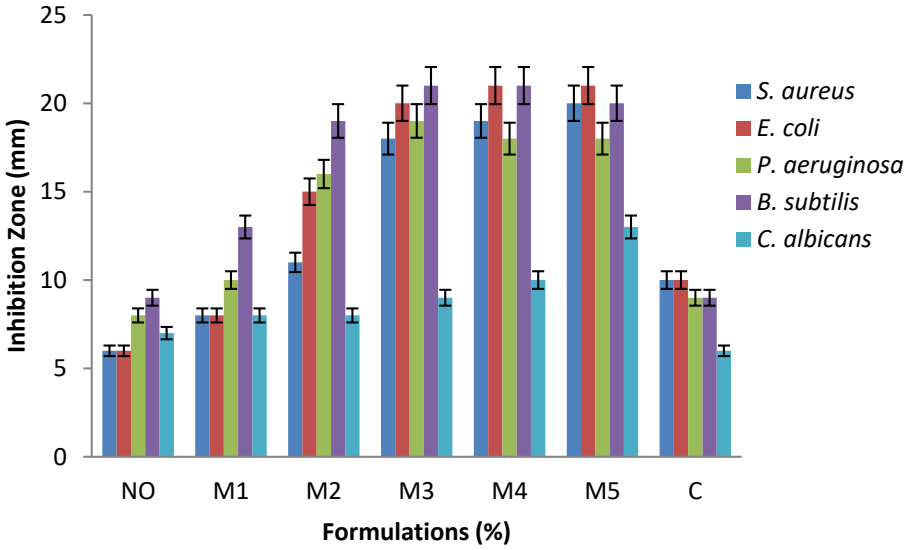

Figure 2: Zone of inhibition (mm) of methanol hand sanitizer formulations

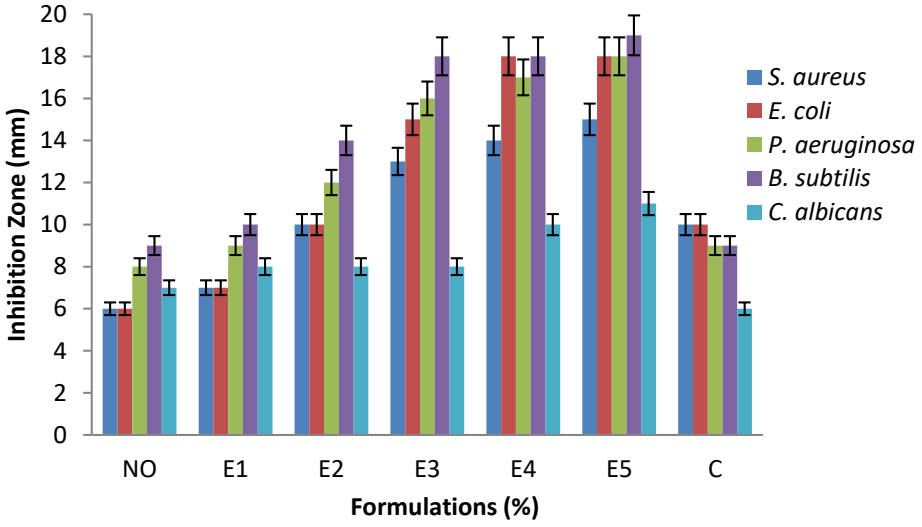

Figure 3: Zone of inhibition (mm) of ethyl acetate extract hand sanitizer formulations

CONCLUSION

This study justified the antimicrobial activity of *S. grandifolia* as reported in literature based on its ethnomedicinal use in the treatment of infectious diseases. The plant would likely be safe when included in hand sanitizers since presently there is no reported adverse consequences with its use in ethnomedicine. It was observed that the antimicrobial activity of all the extract was concentration dependent up to a point when activities became almost static, at this point rate of activity declined.

The highest antimicrobial activity was obtained from the formulations containing methanol extract which follows similar trend with the antimicrobial activity of the leaf extract only. At all concentration of the extract, the *S. grandifolia* hand sanitizer formulations were more effective than the alcohol based sanitizer formulation. All formulations except those containing 0.5% extract are more effective than the commercially available sanitizer.

In conclusion, the incorporation of *S. grandifolia* extract synergized the antimicrobial activity of alcohol only based hand sanitizers which could be used to reduce the spread of infectious pathogens in health care settings.

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Competing Interest

Authors have declared no competing interest.

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تقييم النشاط المضاد للميكروبات لـ *Strombosia grandifolia* Hook.f. خلاصة بنث السابقة تركيبة مطهر اليد

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ملخص.

اليد عامل سهل لانتشار وانتقال مسببات الأمراض والالتهابات المكتسبة من المجتمع. تهدف هذه الدراسة إلى تقييم النشاط المضاد للميكروبات لمعقم اليدين Strombosia grandifolia مقارنة بمطهر الأيدي الكحولي المتاح تجاريًا. تم استخدام الماء والميثانول ومستخلص أسيتات الإيثيل من ورقة سترومبوزيا غراندیفولیا لتكوين معقم لليدين. تم إجراء النشاط المضاد للميكروبات لمطهرات اليدين ضد المكورات العنقودية الذهبية، الزائفة الزنجارية، الإشريكية القولونية، العصوية الرقيقة والمبيضات البيضاء وتم تحليلها باستخدام ANOVA أحادي الاتجاه. كانت المطهرات ناعمة مع شعور بالبرودة على الجلد. كان للمطهر المائي وخلاصة الميثانول نشاط مضاد للميكروبات أفضل بشكل ملحوظ عند مقارنته بمستخلص أسيتات الإيثيل بترتيب حساسية للكائنات الدقيقة مثل Staphylococcus aureus> Bacillus subtilis> Pseudomonas aeruginosa> Escherichia coli> Candida albicans. أدى دمج المستخلص إلى تضافر النشاط المضاد للميكروبات لمطهرات اليد التي تحتوي على الكحول.

الكلمات الدالة: سترومبوزيا غراندیفولیا، نشاط مضاد للميكروبات، فحص كيميائي نباتي، معقم لليدين.

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Antioxidant and Cytotoxic Activity of *Lentinus fasciatus*

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ABSTRACT

The genus *Lentinus* is one of the most studied and medicinally significant groups among mushrooms. *Lentinus fasciatus* is a considerably understudied species and a methanolic formulation was evaluated for its medicinal potential. Phytochemical analysis unveiled the presence of a high amount of phenolic substances in the methanolic extract of the basidiocarps of *L. fasciatus*. The extracted fraction showed notable scavenging properties in in-vitro antioxidant property estimation assays. In the ABTS and DPPH assay, respectively, the EC_{50} values were 332.12 and 180.78 µg/mL. The mushroom extract was also screened for cytotoxic activity against the human breast adenocarcinoma cells (MCF-7). The biocidal activity against cancer cells is further proven by the low LD_{50} value of 246 g/mL in the WST-1 experiment. The background mechanism behind the cytotoxicity was predicted to be mediated by the apoptotic pathways.

Keywords: Antioxidant property, cytotoxicity, free radicals, methanolic extract, mushroom.

INTRODUCTION

Free radicals are recognized to be potentially harmful chemical entities having one or more unpaired electrons. These electrically charged molecules attempt to neutralize themselves by oxidizing other compounds [1]. ROS is the most abundantly produced by products of metabolic processes [2]. The main target of these reactive molecules includes nucleic acids, proteins, lipids, and structural carbohydrates. ROS causes oxidation of proteins and RNA, lesions on DNA strands, alterations in polyunsaturated fatty acids in membranes, mitochondrial depolarization, apoptosis, and many more deleterious effects [3]. A proper balance between the production of oxidants and antioxidants is maintained in healthy living beings. An unhealthy lifestyle, environmental pollution and other factors are forcefully disrupting this balance, for which dietary supplementation of antioxidants is becoming a necessity these days.

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ), propyl gallate (PG) are some of the widely used chemically synthesized antioxidants in the food and medicine industry [4]. Although synthetic antioxidants are in extensive use, safety issues have arisen over time. Long-term intake of these substances can cause major health problems and, in certain circumstances, increase the risk of cancer. BHT has already been shown to be carcinogenic at higher dosages.[5] Thus, there is a growing demand for naturally derived antioxidants with less side effects [6].

Mushrooms have been known to be a treasury of natural bioactive compounds and usage of mushrooms for therapeutic purposes dates back to the Neolithic age. Decades of research have established mushrooms are rich in different kinds of bioactive phytochemicals that are slowly being proved to be effective against several human ailments [6–9]. Most of the species among the genus Lentinus are edible but investigation on their medicinal properties is scarce. Recently, an edible mushroom *Lentinus fasciatus*

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was collected from West Bengal, India [10], whose medicinal importance has not been widely explored. In this article, we have reported the mycochemical composition, antioxidant and cytotoxic efficacy of the methanolic extract of *Lentinus fasciatus*.

MATERIAL AND METHODS

Sample Preparation

Fresh fruit bodies of *Lentinus fasciatus*, naturally grown on the roadside wooden logs, were collected from Barasat, West Bengal, India. The mushroom was identified and authenticated using standard literature [10]. Collected basidiocarps were dried at 40°C for 48h and grinded using an electronic grinder and passed through 160 mesh and stored in a sealed container.

Extraction

Five grams of powdered sample was extracted with methanol for 24h with agitation at regular intervals and this was repeated twice with the residue. The methanolic extract was evaporated to dryness and redissolved in dimethyl sulfoxide (DMSO) to get a stock solution of 500 mg/mL. The stock solution was dissolved further, according to the requirement of the experiments.

Phytochemical analysis

Folin-Ciocalteau reagent was used to measure the total phenolic content [11]. Total phenolic content is represented as µg of gallic acid equivalent (GAE) / mg of extract. Total flavonoid was evaluated according to a customary protocol [12] followed by comparing it with quercetin standard curve. Similarly, the flavonoid content is expressed as µg of quercetin equivalent (QE) / mg of extract. For estimation of β-Carotene and lycopene content 100µL of the extract was taken in a test tube and mixed with 10 mL of acetone and hexane (4:6). The absorbance of the mixture was measured at 453, 505 and 663 nm and the β-Carotene and lycopene content were estimated using the given formula (Nagata and Yamashita, 1992).

$$\beta - \text{Carotene (mg/100mL)} = (0.0458 \times A_{663}) + (0.373 \times A_{505}) - (0.0806 \times A_{453})$$

$$\text{Lycopene (mg/100mL)} = (0.216 \times A_{663}) - (0.304 \times A_{505}) + (0.452 \times A_{453})$$

Estimation of Antioxidant Potential

The total antioxidant capacity of the methanolic extract was estimated according to the following protocol [14]. Total antioxidant capacity is indicated in form of µg of ascorbic acid equivalent (AAE) / mg of extract. To assess the DPPH free radical quenching activity, the methodology described by Pereira et. al. was followed with slight modifications [15]. Reactions were carried out on 96 well plate and the reaction volume was kept at 200 µL. Reaction mixtures consisting of gradually increased concentration of the sample extract and DPPH solution were incubated for 30 minutes. The absorbance of the mixtures was checked at 517nm using a microplate reader (iMark™ Microplate Absorbance Reader, BIO-RAD, USA) to calculate the scavenging activity. EC_{50} value was determined which specifies the concentration of extract at which 50% scavenging of the free radicals took place. ABTS free radical scavenging activity of the extracted fraction was determined according to the protocol standardized by Khatua et. al. (2013) [16]. Similar to DPPH free radical scavenging assay the EC_{50} value was computed from the graphical curve. For authentication, the obtained data of DPPH and ABTS assay was compared to ascorbic acid and trolox respectively.

Cell Culture

Human breast adenocarcinoma cell line MCF-7, obtained from the cell line repository at National Centre for Cell Science, Pune was maintained in Minimum essential medium (***MEM***) supplemented with 10% Foetal Bovine Serum. For optimal growth, the cells were incubated in a humified incubator (CO_2 Incubator, Esco Micro Pte Ltd, Singapore) at 37 °C with 5% CO_2. Trypsin-EDTA was used to dissociate the confluent cells and the

cells were seeded in variable plates for experiments and incubated for 24 hours. The amount of DMSO was restricted to <1% during treatment.

Invitro cytotoxicity assay

MCF-7 cells were seeded in a 96-well microtiter plate and incubated for 24 hours to obtain the cells at the log phase. Treatment of different concentrations of the extract was given to the cells and again incubated for 24h. After that, 10 µL WST-1 reagent (TaKaRa, Japan) was added to each well containing the treated cells and incubated for 2h. Absorbance was measured according to the manufacturer's protocol using a plate reader. The reduction in absorbance was analyzed to determine the cell death percentage at different concentrations and LD_{50} value.

Detection of Apoptosis

Treated cells were stained with acridine orange (AO, 5 mg/mL) and ethidium bromide (EB, 3 mg/mL) and photographed under a fluorescence microscope (Floid Imaging Station, Life Technologies, Waltham, MA, USA). Dead cells produced a brilliant orange fluorescence due to the uptake of EB, while the live cells appeared green due to the uptake of AO.

To further confirm the nature of cell death, nuclear morphology was also characterized using the DAPI staining methodology. After washing the treated cells with phosphate-buffered saline (PBS), the cells were treated with 6-diamidino-2-phenylindole (DAPI) (1µg/mL in PBS). After 15 minutes the washed cells were observed under a fluorescence microscope for nuclear abnormalities.

Statistical analysis

All data are exhibited as the mean ± SD of "n" independent measurements as indicated in the corresponding figure legends. Statistical comparisons were calculated using Student's t-test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Crude organic extracts contain a complex mixture of phenolic compounds which are soluble only in certain organic solvents. A literature survey has confirmed that methanol extraction is one of the most efficient extraction procedures to pull out the phenol-rich fraction from mushroom samples as Methanol represents a higher polarity index than ethanol, ethyl acetate, acetone and other organic solvents [17].

Phytochemical analysis

In this study, methanolic extract of *Lentinus fasciatus* was prepared to quantify some of the significant bio-active chemical groups to confirm the medicinal potential of this mushroom. A yellowish-brown organic formulation was obtained with a high extractive yield of 12.51%. The prime chemical group in the methanolic extract of *L. fasciatus* was found to be phenol. It is a well-established phenomenon that phenolic compounds tend to reduce potential oxidant species due to the presence of the aromatic ring. Besides their antioxidant properties phenols are good antibacterial, antiviral, anti-carcinogenic and anti-mutagenic agents [18]. β-carotene and lycopene are the two main fungal carotenoids, known for their antioxidant activities [19]. Data validates that phenols and flavonoids are the key components that are putative antioxidants, whereas β-carotene and lycopene were found in trace amounts. The total phenol, flavonoid, β-carotene, and lycopene content is summarised in Table 1.

Table 1: Proximate phytochemical composition of the methanol soluble fraction of *L. fasciatus.*

Extractive yield	Total phenols (µg GAE / mg of extract)	Flavonoids (µg QE / mg of extract)	β-Carotene (µg/mg of extract)	Lycopene (µg/mg of extract)
12.51 %	60.894 ± 2.29	5.54 ± 0.84	0.1344 ± 0.006	0.0987 ± 0.006

Antioxidant assays

Antioxidants can counteract various types of radicals or oxidants in several different ways. Thus, to evaluate the antioxidant potential of the methanolic extract of *L. fasciatus*, a series of assays were performed. Phosphomolybdenum method gives a good measure of the antioxidant activity of crude extracts. Mo(IV) is reduced to Mo(V) by antioxidant species in acidic pH and a green phosphate/Mo(V) compound with absorption maxima at 695nm is formed [14]. By spectrometric analysis, the total antioxidant capacity of the methanolic extract was found to be 36.025 ± 0.601 µg AAE/ mg of extract.

ABTS assay is one of the simplest and reliable assays to assess the antioxidant property of biological samples. $ABTS^{\bullet+}$ is produced by oxidizing ABTS with potassium persulfate. The dark bluish-green colour is neutralized upon reduction of $ABTS^{\bullet+}$ by hydrogen donating antioxidants and the measurement of photometric change gives an accurate indication of the antioxidant activity of the sample [20]. The experimental extract was found to be a notable scavenger as it showed quite a significant scavenging activity (18.42%) even at a dose as low as 100 µg/mL. Further, the percentage of scavenging was extended to 34.71, 45.64, 59.70, 70.73 in the presence of the extract at 200, 300, 400, 500 µg/mL respectively (Figure 1). Effective concentration (EC_{50}) at which 50 % scavenging took place was found to be 332.122 ± 0.838 µg/mL.

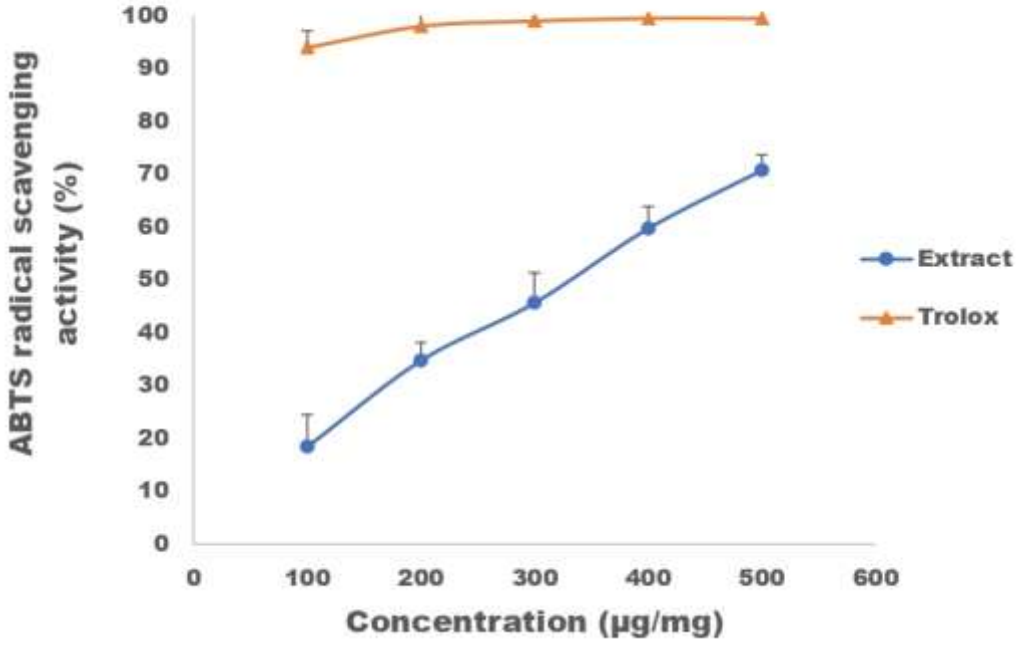

Figure 1: ABTS free radical scavenging activity of the methanolic extract of *L. fasciatus* compared to ascorbic acid as standard. Data are presented as mean ±SEM and are representative of three independent experiments. $p < 0.05$.

Moreover, in order to visualize the scavenging activity in a better way, the extract was tested against DPPH, a commercially available stable free radical that is commonly used to assess the antioxidant property of test samples. The purple colour of the free radical solution gradually fades upon reduction of DPPH radicals by the reducing agents such as antioxidants [15]. The loss of colour gives a photometric measurement of the free radical scavenging property of the test samples. Methanolic extract of *L. fasciatus* showed considerable scavenging property that surged up to 77.1 % at the higher dose of 300 µg/mL (Figure 2). EC_{50} value against DPPH was found to be 180.78 ± 4.04 µg/mL which is lower than the published reports [21] of other *Lentinus* species like *L. squarrosulus, L sajor-caju*. The extracted fraction of the mushroom showed potential antioxidant activity *in-vitro*, indicating that it is an effective antioxidant (Table 2).

Table 2: Antioxidant and Cytotoxic activity of methanolic extract of *L. fasciatus*

Total Antioxidant Capacity	EC_{50} value for ABTS free radical scavenging	EC_{50} value for DPPH free radical scavenging	LD_{50} value of Cytotoxic activity against MCF-7 cells.
36.025 ± 0.601 µg Ascorbic Acid Equivalent (AAE)/ mg of extract	332.122 ± 0.838 µg/mL	180.78 ± 4.04 µg/mL	246 ± 1.045 µg/mL

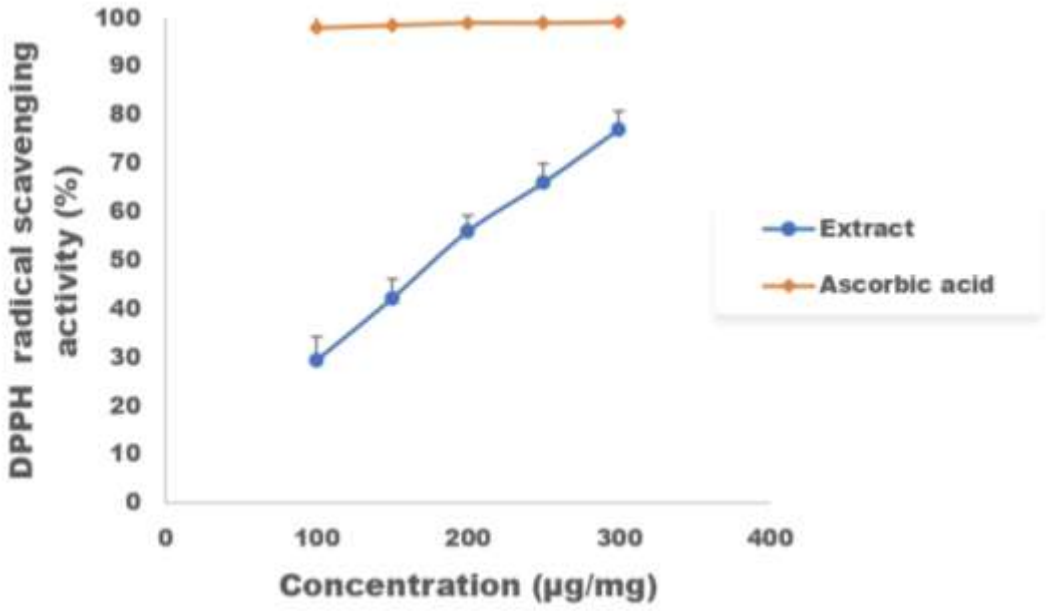

Figure 2: DPPH radical scavenging activity of the methanolic extract of *L. fasciatus* compared with standard. Data are presented as mean ±SEM and are representative of three independent experiments. p < 0.05.

Cytotoxicity assessment

Extensive research established that there is a close correlation between oxidative stress and cancer. Excessive generation of reactive oxygen species stimulates the uncontrolled growth of cells leading to the development of tumours and initiating the process of carcinogenesis [22].

Furthermore, oxidative stress weakens the body's innate antioxidant defence system against angiogenesis and metastasis [23]. Therefore, after getting a positive response in the antioxidant assays, the extracted fraction was further screened for cytotoxic effects on a breast cancer cell line.

To assess the cytotoxic effects of the extract the treated MCF-7 cells were stained with WST-1 regent and orange-colored formazan was produced in proportion to the presence of living cells. WST-1 assay aided us to calculate the proportion of cells that died at various doses of the extract and determining its LD50. At 500 g/mL, 82.56 percent of cancerous cells died, evidencing that the extract is effective in killing cancerous cells (Figure 3). The LD50 value was obtained to be 246 ± 1.045 µg/mL.

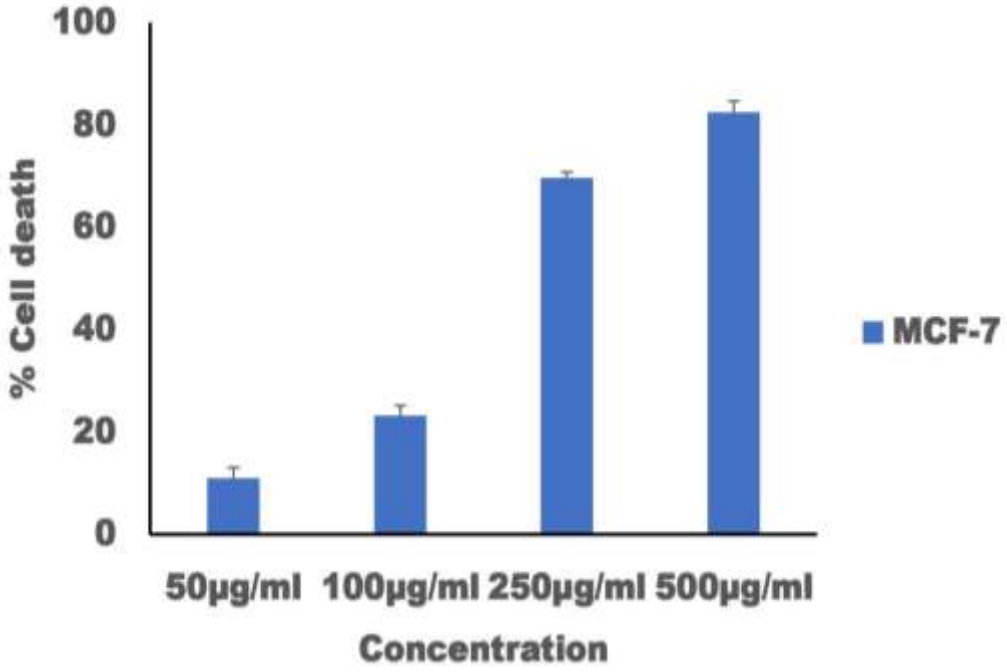

Figure 3: Percentage of cell death in MCF-7 cells induced by the methanolic extract of *L. fasciatus*. Data are presented as mean ±SEM and are representative of three independent experiments. $p < 0.05$.

To visually differentiate between live and dead cells, dual staining with EB and AO was executed. AO can penetrate all the cells independent of their viability but EB cannot make their way into the cells until the integrity of the plasma membrane and the nuclear membrane is lost downstream of death signals [24]. The majority of the treated cells showed orange fluorescence due to the intercalation of EB into their DNA, whereas all cells in the control set were fluorescing only green (Figure 4A). Loss of membrane integrity is also a hallmark of induction of apoptosis.

Apoptosis or programmed cell death is a pathway that entails a series of phenotypic changes in the cell undergoing apoptosis, among which one of the most important attributes being nuclear shrinkage and disintegration [25]. To confirm whether the cytotoxicity imparted by the extract is by inducing apoptosis, DAPI, a fluorescent dye that intercalates into the grooves of DNA, was used to stain the nucleus. Nuclear deformities including shrinkage, blebbing and fragmentation were

observed in most of the treated cells whereas untreated cells portrayed uniform chromatin staining. The observed changes in the nucleus (Figure 4C) indicates that the cell death was possibly mediated by the apoptotic pathway.

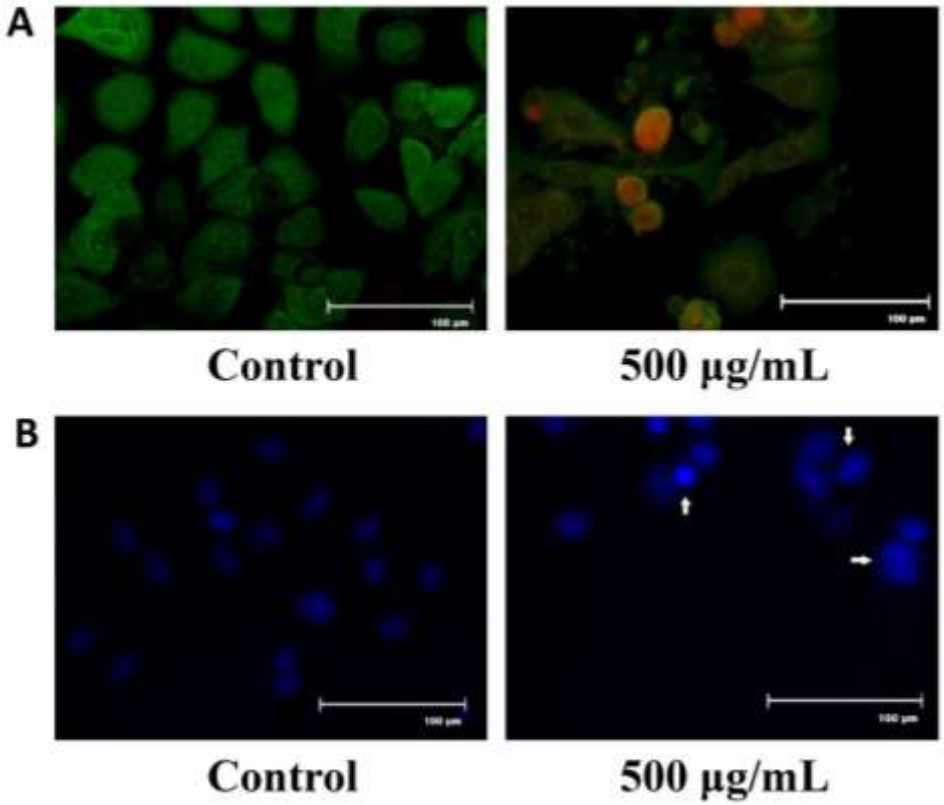

Figure 4: (A) Representation AO/EB dual stained cells after treatment for 24h. (B) DAPI stained cells after treatment for 24h. Images taken under 20X magnification, representing the best of the replicates (n = 3).

CONCLUSION

The methanolic extract of *Lentinus fasciatus* has considerable antioxidant properties, as shown in the findings of this investigation. The crude extract is rich in antioxidant components including phenol and flavonoids as revealed by the quantitative phytochemical assessments. In addition, the cytotoxic property against human cancer cell line displays potential as a possible anti-cancer formulation. Although an early indication of apoptosis was observed in the cancer cells, the underlying mechanism needs further validation. Overall, this mushroom can become an important addition to the future pharmaceutics.

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نشاط مضادات الأكسدة والسمية للخلايا في *Lentinus fasciatus*

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ملخص

يعتبر جنس Lentinus من أكثر المجموعات التي تمت دراستها وذات الأهمية الطبية بين عيش الغراب. Lentinus fasciatus هو نوع غير مدروس بشكل كبير وتم تقييم تركيبة ميثانولية لإمكاناته الطبية. كشف التحليل الكيميائي النباتي النقاب عن وجود كمية عالية من المواد الفينولية في المستخلص الميثانولي من الباسيدوكاربس من L. fasciatus. أظهر الجزء المستخرج خصائص كسح ملحوظة في مقايسات تقدير خصائص مضادات الأكسدة في المختبر. في اختبار ABTS و DPPH، على التوالي، كانت قيم EC50 332.12 و 180.78 ميكروغرام / مل. تم فحص مستخلص الفطر أيضًا بحثًا عن النشاط السام للخلايا ضد خلايا سرطان الثدي البشري (MCF-7). تم إثبات نشاط المبيدات الحيوية ضد الخلايا السرطانية من خلال قيمة LD50 المنخفضة البالغة 246 جم / مل في تجربة WST-1. تم توقع آلية الخلفية وراء السمية الخلوية بواسطة مسارات موت الخلايا المبرمج.

الكلمات الدالة: خاصية مضادات الأكسدة، السمية الخلوية، الجذور الحرة، خلاصة الميثانول، الفطر.

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Knowledge and Practices of Disinfectants and Sanitizers Use during COVID-19 Pandemic in Jordan

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ABSTRACT

Background: The use of antimicrobials has been expanded during the COVID-19 pandemic. This study aims to assess the knowledge and practices of disinfectants and sanitizers use among Jordanian people during the (COVID-19) pandemic.

Methods*:* A web-based cross-sectional descriptive questionnaire was distributed across Jordan between August and September 2020. The questionnaire consisted of three sections inquiring about demographics and general characteristics of the surveyed sample, evaluating the respondents' knowledge about disinfectants, as well as respondents' practices. The questionnaire was completed by 403 literate adult respondents.

Results: Our results indicate that Jordanian adults have used disinfectants increasingly during the COVID-19 outbreak. Knowledge of our study sample was considerably affected by gender ($p=0.044$), income ($p=0.001$), and profession ($p<0.001$). 80.8% of those participants reported skin-related side effects due to disinfectant use during the pandemic. The most used disinfectants were ethanol, followed by soap and water. Generally, study respondents showed positive practices toward the use of disinfectants during the time of the pandemic with few high-risk practices reported. Interestingly, the positive practices applied by Jordanian adults were minimally and not significantly affected by the knowledge about antimicrobials' safe and effective use.

Conclusions: There is an urgent need for a structured effort to increase public awareness regarding the safe and effective use of disinfectants against SARS-CoV-2 transmission.

Keywords: COVID-19, disinfectants, side effects, practices, knowledge.

What is already known about this topic?

To our knowledge, this is the first report in the literature addressing the impact of COVID-19 pandemic on disinfectants and sanitizers (antimicrobials) use among people in a developing country in the Middle East.

What does this article add?

This study was designed to address the knowledge, attitudes, and opinions on disinfectants and sanitizers (antimicrobials) use in developing countries, with Jordan as an example. Our analysis show that structured efforts are urgently needed to increase public awareness regarding the safe and effective use of antimicrobials against SARS-CoV-2 transmission.

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BACKGROUND

Coronaviruses are members of the family Coronaviridae [1, 2]. The novel coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [3]. These viruses are zoonotic pathogens with high mutation rates that are present in humans and various animals [1]. Covid-19 disease could be presented in a wide range of clinical features ranging from an asymptomatic course the to requirement of hospitalization in the intensive care unit causing infections in respiratory, gastrointestinal, hepatic and neurologic systems [4, 5, 6].

SARS-CoV-2 can be transmitted through the air, feces, and soiled surfaces. Furthermore, it can arise on surfaces that are frequently touched [7]. It has been found that human coronaviruses can remain infectious on inanimate surfaces for up to 9 days [8]. Hence, the utmost priority in containing the disease is to prevent the further spread of the virus in public and healthcare settings [8-7.] There are several methods that are currently used to reduce the transmission of the virus, such as the use of masks, social distancing as well as the use of disinfecting and sanitizing agents [7].

Coronaviruses are positive-stranded RNA viruses with an envelope containing glycoprotein spikes, with the largest genomes among RNA viruses [9-10]. Disinfectants and biocides effective against coronaviruses may inactivate the enveloped virus because of their affinity for the lipid-containing viral envelope, the capsid, and the genome [11]. Thereby, the use of antimicrobials and biocides may reduce human- to human- transmission of the virus [12, 13, 14]. There are various types of biocidal agents such as hydrogen peroxide, alcohols, sodium hypochlorite, or benzalkonium chloride that are used worldwide for disinfection, mainly in healthcare settings [8].

According to the United States Environmental Protection Agency (EPA), disinfectants are substances, or mixtures of these substances, used to destroy or suppress the growth of harmful microorganisms, such as bacteria, viruses, or fungi on inanimate objects and surfaces. Several factors should be considered for disinfectants to be effective. For instance, the antimicrobial adequate concentration, and exposure time with the surface, as well as being extremely cautious regarding safety issues [8]. Safety should be a matter of high consideration when dealing with disinfectants in order to prevent hazardous events resulting from the misuse of these chemicals, which can be achieved by the selection of the appropriate disinfectants for its specific surface, minimizing their contact with any body parts, and properly following the manufacturer's instructions. Lack of awareness of using antimicrobials results in side effects. For example, inadequate use in poor ventilation may result in fire, explosion, gas poisoning, or equipment erosion [3]. Moreover, inadequate use of antimicrobials may cause or aggravate asthma in health care settings because of their sensitizing or irritant properties [15, 16, 17]. In addition, dermatitis and other adverse skin effects have been reported among hospital cleaning workers [18].

The aim of this study is to investigate the knowledge and practices of disinfectants and sanitizers use among Jordanian people during the COVID-19 pandemic. To the best of our knowledge, this is the first report from a Middle Eastern country that discusses and investigates deeper factors affecting the knowledge and practices of the Jordanian people when dealing with disinfectants and sanitizers, especially during the COVID-19 pandemic.

METHODS

Instrument and Data Collection

Responses for this study were collected using a web-based cross-sectional questionnaire. A three-section questionnaire was developed by the authors based on the careful and precise observation of the community and a review of recent available literature [19- 31]. The first section of the questionnaire is composed of items related to demographics and general characteristics of the surveyed sample, including gender, age, marital status, governorate of residence, education, monthly income, and profession.

The second section was used to evaluate the respondents' knowledge of disinfectants, including surface disinfectants and sanitizers. In the third section, respondents' practices were investigated.

The knowledge section (section two) consisted of eleven items on a 3-point Likert scale ranging from 1 (disagree) to 3 (agree) and was scored accordingly. The total score of participants' knowledge about disinfectants ranged from 11-33; based on this, participants were divided into poor to moderate knowledge participants (score range 11-28) and high knowledge participants (score range 29-33).

The questionnaire was administered in modern standard Arabic (the official national language in Jordan) utilizing Google form ™ to design the survey which was disseminated to the Jordanian people using emails, personal contact restricted only to relatives and family members, and various social media platforms (e.g., Facebook® Groups, Facebook® Messenger, and WhatsApp) to allow ease of access and reach during the lockdown to the various layers of the Jordanian community.

The questionnaire was supplemented with a protocol, a short introduction, and instructions to participants. Participants who were interested to enroll in this study signed informed consent (electronically) before responding to the questionnaire. The inclusion criteria were age ≥ 18 years, capable of reading and understanding Arabic (literate), and willingness to participate voluntarily. Data collection took place over one month in August and September 2020.

The data collection tool was evaluated, initially, by an expert in the field of pharmaceutical microbiology, based on which the tool was extensively reviewed, this was followed by a pilot testing where 18 responses from randomly selected participants were collected, and the tool was modified accordingly.

Statistical confirmation of validity and reliability was achieved through the calculation of Cronbach' Alpha value which was found to be 0.741 consistency of the tool and Intraclass correlation (ICC) coefficient of 0.732 (95% CI: 0.683-0.760). Sampling adequacy was also confirmed using Principal Components Analysis (PCA) with Kaiser-Meyer-Olkin (KMO) equal to 0.708 and a significant Bartlett's Test ($p<0.000$).

To minimize social desirability bias, assurance was given to participants that the information they provide will be confidential and their identity will stay anonymous. The collected data were encrypted and stored with the corresponding author and further analysis was done anonymously.

Statistical evaluation

Sample size calculation was based on Raosoft™ sample size calculator with a 95% Confidence level, showing a sample size of 385 respondents would be enough. Categorical variables were presented as frequency and percentage and continuous variables as mean and standard deviation, then the Chi-square test was used to test factors that may affect being part of high-knowledge or low-knowledge participants. Further testing of significant factors was done using logistic regression. All hypothesis testing was two-sided. A p-value of < 0.05 was considered significant. Data analysis was performed using SPSS® 23.0 (IBM, Armonk, NY).

RESULTS

A total of 403 Jordanians participated in the present study; 62.5% were females and 37.5% were males. The mean age was 30.1±10.9 years. More than 90% of respondents were residents of the central and northern regions where most COVID-19 virus cases were reported. The majority of the respondents (85.3%) had at least a bachelor's degree. The income of more than half of the respondents was less than 400 JD and 32.3% of the respondents were health sector professionals. Interestingly, 60% of the respondents used governmental sources also 60% of them used social media to get updates about the COVID-19 virus. Only 18.1% of the respondents

stated that they experienced side effects due to disinfectant use during the pandemic, with 80.8% of those participants reporting skin-related side effects (Table 1).

Table 1: Sample Characteristic, N=403

Variable		mean±SD
Age (mean±SD)		30.1±10.9
		N(%)
Gender	Female	252 (62.5)
	Male	151 (37.5)
Marital Status	Single	253 (62.8)
	Married and others	150 (35.2)
Residency	North region	29 (7.2)
	Central region	335 (83.1)
	South region	39 (9.7)
Education	School degree	24 (6)
	Diploma	35 (8.7)
	Bachelor	279 (69.2)
	Postgraduate	65 (16.1)
Income (JD)	<400	217 (53.8)
	400 – 800	108 (26.8)
	>800	78 (19.4)
Employment	Student	149 (37)
	Employed	176 (43.6)
	Unemployed	78 (19.4)
	Unemployed	33 (8.2)
	Housewife	30 (7.4)
	Retired	15 (3.7)
Profession	Non-health Sector	273 (67.7)
	Health Sector	130 (32.3)
	Pharmacist	70 (53.8)
	Physician	17 (13.1)
	Others	43 (33.1)
Where do respondent stay updated with COVID-19 news[@]	Governmental sources	242 (60)
	International sources (e.g WHO, CDC, …)	125 (31)
	Trusted websites and published studies	186 (46.2)
	Social media	242 (60)
	Not interested in updates	33 (8.2)
Respondents who have experienced side effects due to disinfectants use during COVID-19 pandemic		73 (18.1)
Most frequently reported side effects[$] (N=73)	Hands related side effects (skin dryness, itching, redness, wounds, ..)	59 (80.8)

Variable		mean±SD
	Eczema	25 (34.2)
	Asthma	10 (13.7)
	Respiratory tract infection	5 (6.8)
	Others (Nasal congestion, Thyroid gland problems,..)	7 (9.6)
[@]Valid percent (Values don't sum up to 100%)		
[$]Valid percent		

Respondents had a respected level of knowledge about disinfectants and sanitizers use during the COVID-19 pandemic. The mean knowledge score was 27.5±3.2 with scores range 20-33 points and 50% of respondents had scores of 28 or above out of 33. The most alarming outcomes with regard to respondents' knowledge about disinfectants and sanitizers use were that almost one-quarter of respondents believed that these antimicrobials can increase the immunity against SARS-CoV2, 18.1% of respondents thought that disinfectants and sanitizers were able to cure COVID-19 disease and 73% of respondents stated that all types of disinfectants and sanitizers were ineffective against SARS-CoV2. Comparable percentages (36% and 40.6%) of respondents supposed that both the frequent and excessive use of disinfectants and sanitizers will not reduce effectiveness against SARS-CoV2 (Table 2).

Table 2: Respondents' General Knowledge about Antimicrobials, N=403

	Agree	Neutral	Disagree
1. The main purpose of using disinfectant is to prevent the transmission of COVID-19 virus	331 (82.1)	52 (12.9)	20 (5)
2. The main purpose of using disinfectant is to treat COVID-19 virus infection	73 (18.1)	51 (12.7)	279 (69.2)
3. The main purpose of using disinfectant is to increase immunity toward of COVID-19 virus	94 (23.3)	74 (18.4)	235 (58.3)
4. All disinfectants are effective against COVID-19	45 (45)	101 (25.1)	257 (63.8)
5. Some disinfectants are effective against COVID-19	251 (62.3)	105 (26.1)	47 (11.7)
6. All disinfectants are (not) effective against COVID-19*	30 (7.4)	79 (19.6)	294 (73)
7. It is safe to mix all types of disinfectants	14 (3.5)	40 (9.9)	349 (86.6)
8. It is safe to mix some types of disinfectants	63 (15.6)	127 (31.5)	213 (52.9)
9. All types of disinfectants are safe for kids/children	18 (4.5)	61 (15.1)	324 (80.4)
10. Some types of disinfectants are safe for kids/children	243 (60.3)	106 (26.3)	54 (13.4)
11. The frequent use of disinfectants can lead to reduced effectiveness against viruses especially COVID-19 virus[#]	103 (25.6)	155 (38.5)	145 (36)
12. The excessive use of disinfectants can lead to reduced effectiveness against viruses especially COVID-19 virus[#]	85 (6.2)	154 (38.2)	164 (40.7)
13. All disinfectants have similar effectiveness	25 (6.2)	70 (17.4)	308 (76.4)
14. Some disinfectants have similar effectiveness	243 (60.3)	110 (27.3)	50 (12.4)
* This item was used to measure the internal consistency of the scale			
[#]wasn't included in the scale			

The specific knowledge of respondents about antimicrobials' use-related side effects was also analyzed (Figure 1). As expected, respondents assumed that skin-related side effects (92.8%) and respiratory system-related problems (70%) may be consequences of disinfectants and sanitizers use, with little knowledge about other reported side effects.

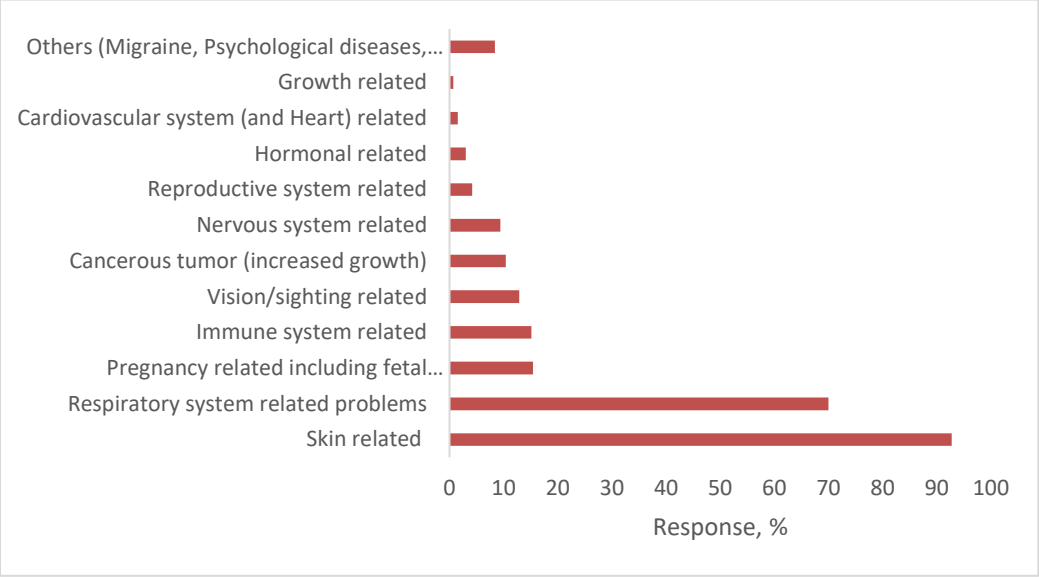

Figure1: Respondents' specific knowledge about disinfectants and sanitizers related side effects

(Table 3) illustrates factors that affect the level of respondents' knowledge about disinfectants and sanitizers use amid the COVID-19 pandemic. Generally, respondents who were female, single, employed, health-care professionals, had a bachelor's degree or more, had relatively low income, are significantly ($p<0.05$) more knowledgeable with regard to the disinfectants and sanitizers use. Further investigation revealed a significant model of the dependent variable (knowledge score) that is considerably affected by gender ($p=0.044$), income ($p=0.001$) and profession ($p<0.001$) (Table 4).

Table 3: Factors that affect Respondents' General Knowledge about Antimicrobials

Variable		Poor to Moderate Knowledge Level, N=280	High Knowledge Level, N=123	*p-value#*
Age		30±11.2	28.8±10.3	0.131
Gender	Female	168 (60)	84 (68.3)	
	Male	112 (40)	39 (31.7)	0.046
Marital Status	Single	167 (59.6)	86 (69.9)	
	Married and others	113 (40.4)	37 (30.1)	0.031
Residency	North region	18 (6.4)	11 (8.9)	
	Central region	229 (81.8)	106 (86.2)	
	South region	33 (11.8)	6 (4.9)	0.076
Education	School degree	19 (6.8)	5 (4.1)	

Variable		Poor to Moderate Knowledge Level, N=280	High Knowledge Level, N=123	*p-value#*
	Diploma	29 (10.4)	6 (4.9)	
	Bachelor	198 (70.70	81 (65.9)	
	Postgraduate	34 (12.1)	31 (25.2)	0.016
Income (JD)	<400	158 (56.4)	59 (48)	
	400 – 800	80 (28.6)	28 (22.8)	
	>800	42 (15)	36 (29.3)	0.004
Employment	Student	96 (34.3)	53 (43.1)	
	Employed	120 (42.9)	56 (45.5)	
	Unemployed	64 (22.9)	14 (14)	0.021
Profession	Non-health Sector	213 (76.1)	61 (49.6)	
	Health Sector	67 (23.9)	62 (50.4)	0.000
# Chi-square test				

Table 4: Logistic Regression Analysis of Factors that Affect Respondents' Knowledge about Antimicrobials - (74.4% Prediction)

Parameter		*p-value*
Model	55.663	0.000
	ExpoB	
Gender (Female/Male)	1.667	0.044
Marital Status	0.604	0.088
Education	1.397	0.100
Income (JD)	1.769	0.001
Employment Status	1.372	0.095
Profession	2.825	0.000

As shown in Figure 2, the vast majority of respondents (94.5%) used ethanol as a disinfectant, followed by soup and water as used by more than 75% of the respondents. However, considerable percentages of the respondents used chloroxylenol, sodium hypochlorite, and acetic acid. Study respondents showed positive practices toward the use of disinfectants and sanitizers during the time of the pandemic (Table 5). In one hand, more than 90% of respondents stated that (1) their consumption of antimicrobials increased during the pandemic, (2) they did encourage others to use antimicrobials, (3) they did ventilate closed places after using antimicrobials, (4) they stored antimicrobial away from children reach and (5) they didn't mix antimicrobials. The good practices continue as more than 80% of the respondents confirmed that they didn't dilute antimicrobials with hot water. On the other hand, only 40.7% of respondents washed vegetables and fruits with antimicrobials and 83.5% of them used vinegar to do so. In the same context, around two-thirds of the respondents read the instructions including storage provided on the package of the antimicrobials. One important outcome of the study is the fact that these positive practices were minimally and not significantly affected by the knowledge about disinfectants and sanitizers.

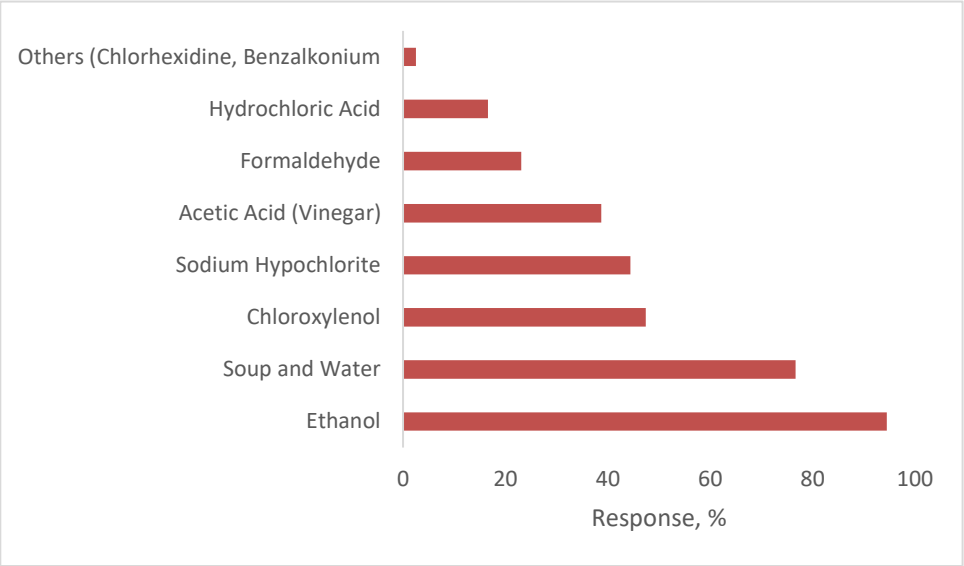

Figure 2: Respondents' most frequently used type of Antimicrobials

Table 5: General Practices of Respondents toward the Safe use of Antimicrobials, N=403

Practice	N (%)	*p-value**
1. Do you encourage others to use disinfectants	377 (93.5)	0.555
2. Do you mix disinfectants	26 (6.5)	0.304
3. Do you dilute disinfectants with hot water	79 (19.6)	0.279
4. Do you ventilate closed places (house, office) after using chemical disinfectants	364 (90.3)	0.792
5. Do you read use instructions (and guide) written on the disinfectant package sticker	278 (69)	0.761
6. Do you read storage instructions (and guide) written on the disinfectant package sticker	262 (65)	0.345
7. Did your consumptions of disinfectant increased because of COVID-19 pandemic	371 (92.1)	0.093
8. Do you use gloves or eye goggles when dealing with/using disinfectants	104 (25.8)	0.298
9. Do you add any type of disinfectants to your water supply to prevent or treat COVID-19	24 (6)	0.005
10. Do you place Disinfectants and leave them to do their action for an enough period of time	280 (69.5)	0.434
11. Do you wash Vegetables or Fruits with any type of Disinfectants	164 (40.7)	0.148
Vinegar	137 (83.5)	0.296
Soup	32 (19.5)	
Others (Ethanol, Hypochlorite, Diluted Chloroxylenol, Special pills)	21 (12.8)	
12. Do you use or have used any Drugs to treat any side effects resulting from the use of Disinfectants	73 (18.1)	0.039
13. Do you use Moisturizers or Creams after using Disinfectants	250 (62)	0.709
14. Do you keep Disinfectants far from the Children upon Storage	376 (93.3)	0.430
*chi-square test of the effect of knowledge on respondents' practices		

More in-depth search uncovered that only 57.8% and 30.5% of the respondents used hand sanitizers when eating and using the mobile phone, respectively. The most preferred type of hand sanitizers was gels and liquids (spreadable forms) and the most frequently reported destination to buy these disinfectants was from large stores (Table 6).

Table 6: Respondents' Specific Practices toward the use of Antimicrobials, N=403

Practice	N	%
When do you/I use disinfectants		
When being in public or using public services	335	83.1
When entering/getting house or workplace/ office	318	78.9
When touching/using door handles	268	66.5
When using any tool or touching any surface	237	58.8
When eating	233	57.8
Before and after wearing gloves	183	45.4
When touching my body or others	176	43.7
When touching/holding/dealing with pets	150	37.2
When using my mobile	123	30.5
Others	27	6.7
Most preferred type(s)/form(s) of disinfectant		
Spreadable Forms (Liquid or Gel)	359	89.1
Sprayable Forms (mists)	229	56.8
Solid Form (like soup bars)	225	55.8
Tissue Form (wipes)	204	50.6
Powder	77	19.1
Others	10	2.5
Where do you buy disinfectants		
Large stores (like hypermarkets)	341	84.6
Small stores (like supermarkets)	173	42.9
Pharmacy	258	64
Others (warehouses, home-made by local producers, …)	17	4.2

DISCUSSION

This analysis show that Jordanian adults have taken major precautions during the outbreak and responded by using increasing amounts of antimicrobials compared to their usual consumption as reported by 92.1% of the respondents. Consistent with the general recommendation for regular disinfection of inanimate surfaces and objects, the majority of respondents in this study reported increased use of disinfectants in their houses and workplaces. However, significant percent of the respondents reported high-risk practices such as using ethanol, hypochlorite, and diluted chloroxylenol to wash fruits and vegetables,

mixing disinfectants, diluting disinfectants with hot water, and applying disinfectants and cleaning products to bare skin. Nonetheless, such poor practices may yield hazardous effects, such as tissue damage and injuries [32-35]. and should be firmly eluded.

While the undesirable health effects that were reported by most respondents could not be attributed to their engaging in high-risk practices, the potential association between these hazardous practices and severe health consequences designate the necessity for structured efforts to increase public awareness regarding the safe and effective use of disinfectants and sanitizers against SARS-CoV-2 transmission.

In addition, crucial knowledge gaps in the use of disinfectants and sanitizers among Jordanian adults during the COVID-19 pandemic were also revealed in this report. The prime gaps were apparent in knowledge about the effectiveness of disinfectants and sanitizers to protect against SARS-CoV-2 transmission. Besides, major gaps were found in the knowledge about potential side effects that may be associated with antimicrobial use.

Despite the identified knowledge gaps among Jordanian adults, most study participants demonstrated positive practices that were minimally and not significantly affected by their knowledge about antimicrobials' safe and effective use. This observation may be attributed to the fact that most respondents reported that they tend to read the instruction labels for antimicrobials and disinfectants before use. Thus, filling the knowledge gaps with awareness messages regarding the safe and effective use of disinfectants and sanitizers should promote proper use, reduce side effects, and result in more effective inhibition of SARS-CoV-2 transmission among the Jordanian population.

The findings reported in this study are subject to several limitations. First, although responses were collected from northern, central, and southern districts to be nationally representative of Jordan's demographics, responses of our sample may not be truthfully representative of knowledge and practices shared by the Jordanian population. Second, cross-sectional studies do not permit a direct correlation of specific outcomes, such as hazardous health consequences, to definite knowledge gaps or practices. Lastly, responses were collected at a particular time point and might not reflect the continuing changes in public knowledge and practices throughout the pandemic. Thus, ongoing efforts to collect data over a longer time span should characterize the differences in the knowledge gaps and practices among the Jordanian public.

CONCLUSION

Jordanian adults reported increased use of antimicrobials amid the COVID-19 pandemic with fair knowledge and positive practices reported by most of the study participants. While the level of knowledge was significantly affected by gender, income, and profession, the positive practices reported by Jordanian adults were minimally affected by their knowledge about antimicrobials' safe and effective use. The ability of Jordanian adults to apply positive practices of antimicrobials' use during the pandemic regardless of their knowledge may indicate the value of sending proper awareness messages to emphasize evidence-based and safe practices concerning the use of antimicrobials for COVID-19 prevention.

Lists of Abbreviations

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

EPA: Environmental Protection Agency

ICC: Intraclass correlation

PCA: Principal Components Analysis

KMO: Kaiser-Meyer-Olkin

Declarations

Ethics approval and consent to participate

All participants provided written informed consent.

Consent for publication

Not applicable.

Availability of data and material

Access to the data can be requested by sending an e-mail to S.bardaweel@ju.edu.jo

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SB conceived and designed the study. QM and HM performed the data collection under SA supervision. SM conducted the data analysis. AA prepared the first draft of the manuscript. SB and SA undertook a critical review of the manuscript. All authors provided comments on subsequent drafts and approved the final version of the manuscript.

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المعرفة والممارسات المتعلقة باستخدام المطهرات والمعقمات أثناء جائحة كوفيد-19 في الأردن

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ملخص

الخلفية: تم التوسع في استخدام مضادات الميكروبات خلال جائحة كوفيد-19. تهدف هذه الدراسة إلى تقييم معرفة وممارسات استخدام المطهرات والمعقمات بين الأردنيين خلال جائحة (كوفيد-19).

الطُرق: تم توزيع استبيان وصفي مقطعي على شبكة الإنترنت في جميع أنحاء الأردن خلال شهري أغسطس وسبتمبر من عام 2020. يتكون الاستبيان من ثلاثة أقسام تستفسر عن التركيبة السكانية والخصائص العامة للعينة التي تم مسحها، وتقييم معرفة المستجيبين حول المطهرات، والمعقمات وكذلك ممارسات المستجيبين. تم إكمال الاستبيان من قبل 403 شخصاً بالغاً متعلماً.

النتائج: تشير نتائجنا إلى أن البالغين الأردنيين استخدموا المطهرات بشكل متزايد أثناء تفشي كوفيد-19. تأثرت المعرفة في عينة الدراسة بشكل كبير بالجنس والدخل والمهنة. أبلغ 80.8٪ من المشاركين في الدراسة عن آثار جانبية مرتبطة بالجلد بسبب استخدام المطهرات أثناء الجائحة. وكانت أكثر المطهرات استخدامًا هي الإيثانول، يليه الصابون والماء. بشكل عام، أظهر المشاركون في الدراسة ممارسات إيجابية تجاه استخدام المطهرات خلال وقت الجائحة مع الإبلاغ عن عدد قليل من الممارسات عالية الخطورة. ومن المثير للاهتمام، أن الممارسات الإيجابية التي طبقها الأردنيون لم تتأثر بشكل كبير بمعرفتهم حول الاستخدام الآمن والفعال لمضادات الميكروبات.

الخاتمة: في الختام ، هناك حاجة ملحة لبذل جهود منظمة لزيادة الوعي العام بشأن الاستخدام الآمن والفعال للمطهرات ضد انتقال فيرويس سارس كوف-2.

الكلمات الدالة: كوفيد-19، المطهرات، الآثار الجانبية، الممارسات، المعرفة.

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Identification of Pharmaceutically Important Constituents of Quinoa Root

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ABSTRACT

The present investigation was carried out to explore the bioactive compounds from the *n*-hexane fraction of methanolic extract of quinoa (*Chenopodium quinoa* Willd.) roots. For this purpose, *C. quinoa* roots were collected, shade dried, and crushed into a fine powder. The powdered material was extracted in methanol, filtered, and the filtrate was partitioned with *n*-hexane, followed by GC-MS analysis of the *n*-hexane fraction. The quantitative determination of this fraction revealed the presence of 15 phytochemical constituents of diverse nature. Among these, octadec-9-enoic acid (44.18%); *n*-hexadecanoic acid (18.87%); methyl (Z)-octadec-9-enoate (12.87%); methyl hexadecanoate (4.30%); 2,3-dihydroxypropyl elaidate (3.63%); phthalic acid (3.08%); methyl octadecanoate (2.27%) and 1,12-tridecadiene (2.00%) were prevailing as the most abundant to moderately occurring compounds. A thorough literature survey was carried out to collect information regarding the pharmaceutical properties of the identified compounds. It showed some of the identified compounds namely dodecanoic acid; tetradecanoic acid; 2-benzoyl-d-galactosan; *n*-hexadecanoic acid; methyl octadecanoate; octadec-9-enoic acid; and 2,3-dihydroxypropyl elaidate possess antifungal, antibacterial, antioxidant, antiviral, anti-inflammatory, and/or anti-cancer properties.

Keywords: Bioactive constituents, *Chenopodium quinoa*, n-hexane extract, GC-MS analysis, root.

INTRODUCTION

The plant kingdom represents an extraordinary reservoir of natural bioactive compounds across the globe. These natural products have been exploited to prepare traditional, folk, and modern medical systems.[1,2] Several plant genera have been screened in search of alternatives to reduce the dependency on synthetic substances.[3,4] Many plant species produce organic compounds such as phenolic acid, phenols, flavones, eugenol, epicatechin, carvacrol, quinones, thymol, myricetin, flavonoids, coumarins, tannins, and flavanols.[5,6] Natural plant-based products are gaining importance worldwide because of their non-toxic behavior, fast action, efficiency even at lower concentrations, pleasant odor, and cost-effective properties.[7] In addition, they presumed a preventive role to indicate their diverse beneficial functions against soil-borne fungal pathogens, human pathogens and food-borne diseases.[8-10]

Quinoa (*Chenopodium quinoa* Willd.) is an ancient crop grown widely in South America, Chile, China, Argentina, Bolivia, Ecuador, Colombia, Peru, Canada and France.[11] Recently, it has evoked interest in Asia, especially in Pakistan, because of its richness in protein contents, amino acids, lipids, fibers, minerals (Zn, Cu, Fe, Mg, Ca), and vitamins A and E.[12] It is considered a multipurpose agro-industrial crop consumed in the form of flour, grain, cereals, and cookies.[13] It is cultivated as a potential crop in salt, saline, drought and frost-affected areas.[14] Moreover, it is a disease-resistant, early-maturing plant commonly used as a break crop in a crop rotation system. Quinoa is rich in saponins and other important

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compounds exhibiting various biological activities.[15,16] The present investigation was undertaken to determine the pharmaceutically important compounds from quinoa's n-hexane soluble fraction of methanolic root extract.

MATERIALS AND METHODS

Cultivation of Quinoa

Seeds of Quinoa var. Colorado 407D, with origin from Colorado, USA was sown in autumn 2017 under agro-ecological conditions in Lahore, Pakistan. After the crop matured, the plant roots (2 kg) were carefully collected and washed thoroughly under tap water to remove physical contaminants and then shade dried. After that, the roots were cut into small pieces and completely dried at 40 ºC in an electric oven.

Extract preparation

The dried roots were pulverized into a fine powder by using a mechanical grinder and exhaustively macerated with methanol (6 L) for 15 days at room temperature. The material was then filtered through Whatman No. 1 filter paper. The methanol was evaporated on a rotary evaporator at 45 °C to get a gummy residue (98 g) remaining stirred in 150 mL of distilled water. The resultant mixture was mixed with *n*-hexane (500 mL), thoroughly shaken, put in a separating funnel and left for 2 hours to separate the *n*-hexane layer.[17]

GC-MS analysis

The *n*-hexane fraction was subjected to GC-MS analysis to identify compounds following Khan and Javaid.[18] Analysis was done on a Shimadzu GC-2010plus system coupled with autosampler AOC-20s, an auto-injector AOC-20i, and a gas chromatograph. A capillary column of 0.25 µm × 0.25 mm × 30 m was used in this study. Helium was used as a carrier gas. Turbo mass 5.2 gold Perkin Elmer was used as the mass detector. A 1.0 µl volume of sample was injected by setting the injector at 250 °C. The interface temperature was set at 320 °C. The initial column temperature was 100 °C for 60 s after injection of the sample and enhanced from 100 to 200 °C at 20 °C min^{-1}, and finally from 200 °C to 300 °C at 40 °C min^{-1}. The total run time of the sample was 11 min. Compounds were identified by NIST (National Institute of Standards and Technology) Library.

Literature survey

A thorough survey of the literature was done to search for bioactivities of the various compounds identified through GC-MS. Structures of pharmaceutically important compounds were drawn by using the software ChemDraw.

RESULTS AND DISCUSSION

The GC-MS chromatogram for the quinoa's *n*-hexane soluble fraction of methanolic root extract is given in Figure 1. Results reveal the presence of 15 constituents belonging to diverse groups of natural compounds. Details of the identified compounds with their molecular weight, peak area percentages and retention time are shown in Table 1, whereas the structures of these compounds are given in Figure 2. The most prevailing chemical constituents were octadec-9-enoic acid (**12**); *n*-hexadecanoic acid (**9**); and methyl (Z)-octadec-9-enoate (**10**) with peak areas of 44.18%, 18.87% and 12.87%, respectively. The moderately occurring compounds namely methyl hexadecanoate (**7**); 2,3-dihydroxypropyl elaidate (**14**); phthalic acid (**15**); methyl octadecanoate (**11**) and 1,12-tridecadiene (**8**) were showing 4.30%, 3.63%, 3.08%, 2.27% and 2.00% peak areas, respectively. The compounds present in less concentrations were ar-tumerone, 2-methyl-6-(4-methylphenyl)-2-hepten-4-one (**3**); tetradecanoic acid (**4**); 2-benzoyl-d-galactosan (**6**); pentadecane (**5**); 10,13-eicosadienoic acid, methyl ester (**13**); dodecanoic acid (**1**) and hexadecane (**2**) with peak areas ranging from 1.68 to 0.77%.

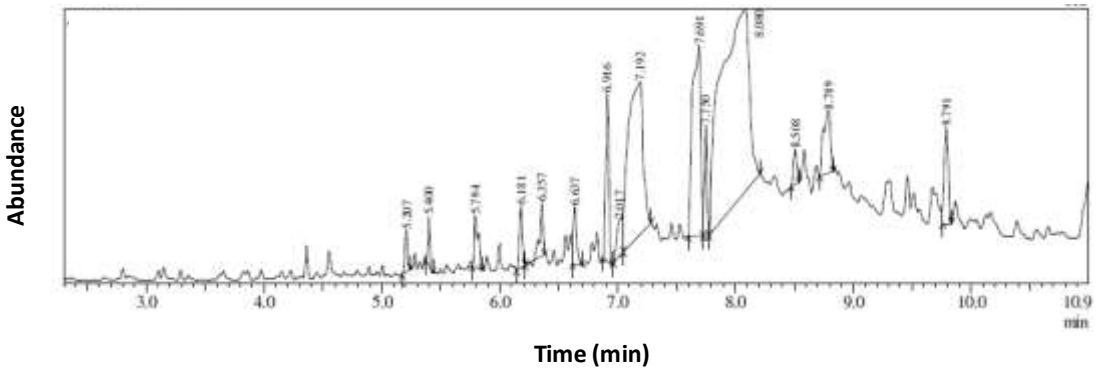

Figure 1: GC-MS chromatogram of *n*-hexane fraction of methanolic extract of *Chenopodium quinoa* roots

Table 1: Compounds identified in *n*-hexane fraction of methanolic extract of quinoa roots through GC-MS analysis.

Sr. No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
1	Dodecanoic acid	$C_{12}H_{24}O_2$	200	5.207	0.92
2	Hexadecane	$C_{16}H_{34}$	226	5.400	0.77
3	Ar-tumerone, 2-Methyl-6-(4-methylphenyl)-2-hepten-4-One	$C_{15}H_{20}O$	216	5.794	1.68
4	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	6.181	1.53
5	Pentadecane	$C_{15}H_{32}$	212	6.357	1.41
6	2-Benzoyl-d-galactosan	$C_{13}H_{14}O_6$	266	6.637	1.51
7	Methyl hexadecanoate	$C_{17}H_{34}O_2$	270	6.916	4.30
8	1,12-Tridecadiene	$C_{13}H_{24}$	180	7.017	2.00
9	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	7.192	18.87
10	Methyl (Z)-octadec-9-enoate	$C_{19}H_{36}O_2$	296	7.691	12.87
11	Methyl octadecanoate	$C_{19}H_{38}O_2$	298	7.750	2.27
12	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	282	8.080	44.18
13	10,13-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322	8.508	0.98
14	2,3-Dihydroxypropyl elaidate	$C_{21}H_{40}O_4$	356	8.789	3.63
15	Phthalic acid	$C_{24}H_{38}O_4$	390	9.791	3.08

Table 2: Bioactivity of components of *n*-hexaane fraction of methanolic extract of quinoa roots.

Comp. No.	Names of compounds	Bioactivity	Reference
1	Dodecanoic acid	Antibacterial, antiviral, antioxidant	[34,35]
2	Hexadecane	Antidiarrheal, antioxidant antimicrobial	[2]
3	Ar-tumerone, 2-Methyl-6-(4-methylphenyl)-2-hepten-4-one	Pharmaceutical and medicinal properties	[32]

Comp. No.	Names of compounds	Bioactivity	Reference
4	Tetradecanoic acid	Anti-inflammatory, antimicrobial	[31]
5	Pentadecane	Antiulcer, antitussive and antioxidant	[20,33]
6	2-Benzoyl-d-galactosan	Antioxidant	[30]
7	Methyl hexadecanoate	Anti-inflammatory, antioxidant, antibacterial	[22]
8	1,12-Tridecadiene	No activity reported	-
9	*n*-Hexadecanoic acid	Antioxidant, antimicrobial	[21]
10	Methyl (Z)-octadec-9-enoate	Antibacterial, antifungal	[27]
11	Methyl octadecanoate	Antibacterial, antiviral	[26]
12	Octadec-9-enoic acid	Anti-inflammatory, cancer preventive, antioxidant	[19,20]
13	10,13-Eicosadienoic acid, methyl ester	Antioxidant	[31]
14	2,3-Dihydroxypropyl elaidate	Anticancer, antimicrobial, antioxidant	[23]
15	Phthalic acid	Antibacterial and antifungal	[28,29]

The most abundant compound **12** is also known as oleic acid, an unsaturated fatty acid previously isolated from a medicinally important plant Tectaria coadunata leaves that possess strong pharmaceutical, anti-inflammatory, cancer preventive, and antioxidant properties.[19,20] Likewise, compounds **2**, **4**, **7**, **9** and **14** have also been reported to possess strong antioxidant, anti-inflammatory, antibacterial, nematicide, antimicrobial and pesticide properties.[21-25] Similarly, in previous studies, compounds **10** and **11** were found effective against pathogenic bacterial strains, including *Staphylococcus epidermidis, Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumoniae, Citrobacter freundi, Salmonella typhi* and *Enterobacter aerogenes*.[26,27] Moreover, compound **15** was identified from a medicinal plant *Hibiscus rosa-sinensis* flower extract. It was tested against gram-positive bacterial strains, namely *Bacillus subtilis* and *Staphylococcus aureus*, and against phytopathogenic fungi *viz. Aspergillus flavus, Drechslera australiensis, Fusarium oxysporum, Alternaria alternata* and *Macrophomina phaseolina*. The compound was very effective in completely inhibiting the tested bacterial and fungal pathogens.[28,29] Similarly, compounds **6** and **13** have been reported from *Homalium zeylanicum* and *Actinidia deliciosa* plant extracts with potent antioxidant activities.[30,31] Likewise, many scientists worked on compounds **1**, **3** and **5** to evaluate their antioxidant, antiviral, antibacterial, pharmaceutical and medicinal efficacy against human diseases, including cancer, ulcer and tussive.[32-35] Therefore, the present study concludes that the *n*-hexane fraction of root extract of *C. quinoa* is enriched with various bioactive substances having antioxidant, antiviral, antifungal, antibacterial, pharmaceutical, pesticidal and anti-inflammatory activities justifying quinoa root as a major source of compounds of pharmaceutical and pesticidal importance.

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تحديد المكونات المهمة صيدلانياً لجذر الكينوا

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ملخص

تم إجراء الدراسة الحالية لاستكشاف المركبات النشطة بيولوجيًا من الجزء hexan-n من المستخلص الميثانولي لجذور الكينوا. (Chenopodium quinoa Willd) لهذا الغرض، تم جمع جذور C. quinoa وتجفيف الظل وسحقها إلى مسحوق ناعم. تم استخلاص مادة المسحوق في ميثانول، وترشيحه، وتم تقسيم المرشح باستخدام hexan-n، متبوعًا بتحليل GC-MS لجزء hexan-n. كشف التحديد الكمي لهذا الجزء عن وجود 15 مكونًا كيميائيًا نباتيًا ذات طبيعة متنوعة. ومن بين هؤلاء octadec-9-enoic acid (44.18٪؛ n-hexadecanoic acid (18.87٪؛ methyl (Z)-octadec-9-enoate (12.87٪؛ methyl hexadecanoate (4.30٪؛ dihydroxypropyl elaidate (3.63–2,3٪؛ phthalic acid (3.08٪؛ methyl octadecanoate (2.27٪؛ و tridecadiene (2.00–1,12٪ (باعتبارها الأكثر وفرة للمركبات المتوسطة التكوّن. تم إجراء مسح شامل للأدبيات لجمع المعلومات المتعلقة بالخصائص الصيدلانية للمركبات المحددة. أظهرت بعض المركبات المحددة وهي n-dodecanoic acid; tetradecanoic acid; 2-benzoyl-d-galactosan; hexadecanoic acid; methyl octadecanoate; octadec-9-enoic acid؛ و dihydroxypropyl elaidate–2،3 يمتلكان خصائص مضادة للفطريات، ومضادة للبكتيريا، ومضادة للأكسدة، ومضادة للفيروسات، ومضادة للالتهابات، و / أو مضادة للسرطان.

الكلمات الدالة: المكونات النشطة بيولوجيًا، تشينوبوديوم كينوا، مستخلص ن–هكسان، تحليل GC-MS، الجذر.

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Comparison between the Efficacy of *Nigella Sativa* Aqueous Extract and Its Oil on Methimazole-induced Hypothyroidism in Albino Mice

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ABSTRACT

Objective: This study was conducted to evaluate the impact of both aqueous extract of *Nigella sativa* and its oil in treating the hypothyroid induced by methimazole in female of white mice in order to determine which one of them is more effective in treating of hypothyroidism, aiming to use it as a natural treatment instead of the chemical treatments which have dangerous side effects.

Materials and Methods: The study included 40 female white mice dived into 4 groups 10 mice each one. Group 1 (control): orally administered and treated with distilled water. Group 2 (HYP): orally treated with methimazole at a dose 0.05-mg/kg/ body weight/day for 3 weeks. Group 3 (NSE): the hypothyroidism was induced as in group 2, then treated orally with aqueous *Nigella sativa* extract at a dose of 400mg/kg/body weight/ day for 4 weeks. Group 4 (NSO): the hypothyroidism was induced as group 2, then treated orally with *Nigella sativa* oil at a dose of 1 ml/kg/body weight/ day for 4 weeks.

Results: This study showed that both NSE and NSO caused a significant decreasing $p<0.05$ of TSH level and a significant increase of FT3 level. Also, NSO caused increasing of FT4 level, while the increase of FT4 was un-significant under NSE treatment.

Conclusions: The efficacy of NSO is higher than the NSE in treating hypothyroidism.

Keywords: Aqueous extract, *Nigella sativa*, FT4, FT3, TSH, Hypothyroidism.

INTRODUCTION

Many medicinal plants have been used in curing illnesses for many years. The whole plant or some parts were used after soaking or boiling without determining the effective material. Due to the development of chemical and pharmaceutical sciences, the chemical effective materials of plants were isolated in order to determine the perfect form to use it (aqueous extract, alcoholic extracts by its various types or oils). Some of these plants are used in curing many diseases instead of chemical medications that have dangerous side effects. The World Health Organization (WHO) has designated medical plants within pharmaceutical safety standards in many countries.

Nigella sativa is seed of herbal plants belonging to Ranunculaceae family known as "Black cumin", its original habitat in the Mediterranean countries, Pakistan, and India [1]. It's rich in nutrients, 1000 g of *N. sativa* contains 210g of protein, 350g of carbohydrate, 350-380g of oils. Many amino acids were isolated and the most important amino acid are tyrosine and lysine [2]. Minerals (Cu, Zn, Se, Fe), vitamins (A, B1, B2, B3, C). It also contains many special secondary metabolites such as alkaloids (Nigellicimine, Nigellicimine-N-oxide, Nigellidine) and saponies [3]. it has also some special oil which contains saturated fatty acids (Linoleic acid, oleic acid) and many unsaturated fatty acids, it's mainly

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concentrated in fixed oil of Nigella Sativa, and also it contains essential oil and violate oil, that has the most effective components of Nigella Sativa ,which are phenols and terpenoid as thymoquinon, thymol, limonene, and p-cymen [4,5], and polyphenols compound as α,β,γ tocopherol (pro-vitamin E)[6] .

Nigella sativa was used in traditional medicine to treat headache, toothache, intestinal warms, nasal congestion, increase milk production, back pain, hypertension and gastrointestinal [7].

Recent studies have indicated to the efficiency *Nigella sativa* aqueous extract analgesic and anti-inflammatory [8], increasing the red blood cells and decreasing the white blood cells with modification in formula of white blood cells [9], Treating diabetes[10] . As well as *Nigella Sativa* oil increases the levels of (LH, FSH) [11], and treats hepatitis-c and non-alcoholic fatty liver disease (NADFL)[12,13], and it has the ability to treat most allergic and respiratory diseases because it inhibits the release of histamine and decreases the production of eosinophil and IL-10 [14].

The thyroid gland is the largest endocrine in the human body, it's controls metabolism [15], and sensitivity to other hormones. As well as regulate the growth and functioning of many other systems in the body[16] . It's secreting three hormones (Thyroxin T4, Triiodothyronine T3, and calcitonin). Thyroid stimulating Hormone produced by anterior lobe of the pituitary gland, regulate the production of hormones (T3, T4), also its under control of (TRH) [17]

Many Studies refereed to the existence of relationship between the use of *N. sativa* and increasing of thyroid gland hormones in blood serum. Where the oral delivery of *N. sativa* ethanol extract to thyroid healthy rats increases the levels of (T3, T4) hormones in blood serum[18]. As well as hydro-alcoholic extract increased the level of thyroid hormones thyroid of healthy white mice female too[19]. Another study clarified that using powdered *N. sativa* increased the level of T3 hormone in which had Hashimoto's thyroiditis [20].

MATERIALS AND METHODS

Experimental animals

In this study 40 adult Albino female mice/ Balb-c were used, with weights between 25-30g, obtained from the Scientific Research center, Damascus at the age 5-6 weeks, were placed in the physiology laboratory at Tishreen University for 4 weeks in order to adapt them to the condition of experiment (were placed in special cages furnished with sawdust, good ventilation, food made of wheat and dried bread and source of water), in addition to the light system(12 hours of lighting and 12 hours of darkness), and temperature of 28-30 c^{o}.

Plant Material

Taxonomic Classification of *Nigella Sativa*

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Ranunculales

Family : Ranunculaceae

Genus: *Nigella*

Species: *N. Sativa*

Plant material of Nigella Sativa Targeted to the aqueous extract was collected from experimental fields of the General Commission for Scientific Agricultural Research, Hama center.

Material was preserved in paper bags for further Extraction process .

Preparation of extract and oil *Nigella sativa*

-The aqueous extract of *Nigella sativa* was prepared according to the Herndez method [21]:

Seeds were crushed by the grinder, then 20g of seeds powder was added to 400ml of distilled water, it was mixed with a magnetic mixer for an hour, the mixture was left for 24 hours at laboratory room temperature, the mixture was then filtered using several layers of medical gauze to get rid of residue, the filtrate was distributed in plastic tubes, it was centrifuged at 300 rpm for 10 minutes,

the filtrate was taken and filtered again using filter papers which permeability is 0.01, the product was dried in the oven and then kept in the refrigerator until use.

-The *Nigella Sativa* oil:

The used oil was extracted using pressure and cooling method of seeds collected from local market of Hama city.

Experimentally induced hypothyroidism

Animals were received methimazole (TABAZOL) anti thyroid drug at dose of 0,05 mg/kg body weight / day for successive 3 week [22].

Experimental design

40 female mice were divided into 4 groups each group consisted 10 mice.

Group 1 (cont.): control group was treated orally with distilled water at a dose of 0.05 mg/kg/body weight /day for 3 week.

Group 2 (HYP): Hypothyroidism group, hypothyroidism was induced by oral delivery of methimazole at dose of 0.05 mg/kg/body weight/day for 3 weeks

Group 3 (NSA): hypothyroidism was induced as in group 2, and then it was treated orally with aqueous extract at dose of 400 mg/kg/ body weight/day for 4 weeks.

Group 4 (NSO): hypothyroidism was induced as in group 2, and then it was treated orally with oil at dose of 1ml /kg/ body weight/day for 4 weeks.

Sample collection

Animals in 4th group were killed with chloroform and blood sample were taken from heart by cardiac puncture. Plasma was separated by centrifuge at speed 3000 rpm for 30 minutes then placed in plain containers and stored at 4^{o}c until analysis.

Hormone estimation

The levels of TSH, FT4 and FT3 were determined using the Siemens® Kit, Siemens healthcare diagnostics products Ltd, UK by IMMULITE- 1000/ Siemens® , the device uses the immunoassay analyzer system, which based on competitive binding of hormones.

Results:

Table (1): Thyroid function tests before and after supplementation of *Nigella Sativa*

Group	TSH ulU/ml	FT3 pg/dl	FT4 g/dl
Group 1 (cont)	0.12±0.03 A	C 412.20±75.80	C 1.93±0.56
Group 2 (HYT)	B 2.13±1.03	A 191.90±49	A 0.82±0.14
Group 3 (NSA)	A 0.42±0.01	B 339.80±34.43	AB 1.02±0.35
Group 4 (NSO)	A 0.05±0.02	B 341.20±54.51	B 1.20±0.23
LSD 5%	0.48	50.89	0.33

Values are the means ± S.D. (n = 10). Group 1: control mice administered distilled water, Group 2: administered Methimazole 0.05 mg/kg/day, Group 3: administered *Nigella Sativa* aqueous Extract 400mg/kg/day, Group 4: administered *Nigella Sativa* oil 1ml/kg/day.

Comparison of the means value of TSH in studied group:

Table (1) and figure (1) show a significant increase occurring ($p<0.05$) means value of TSH after induced hypothyroidism and significantly decrease after the treatment by *Nigella Sativa* aqueous extract and *Nigella Sativa* oil. There were no significant differences compared to the control group and group 3 and group 4, this means that aqueous extract and oil of *Nigella sativa* restored to semi normal value to control.

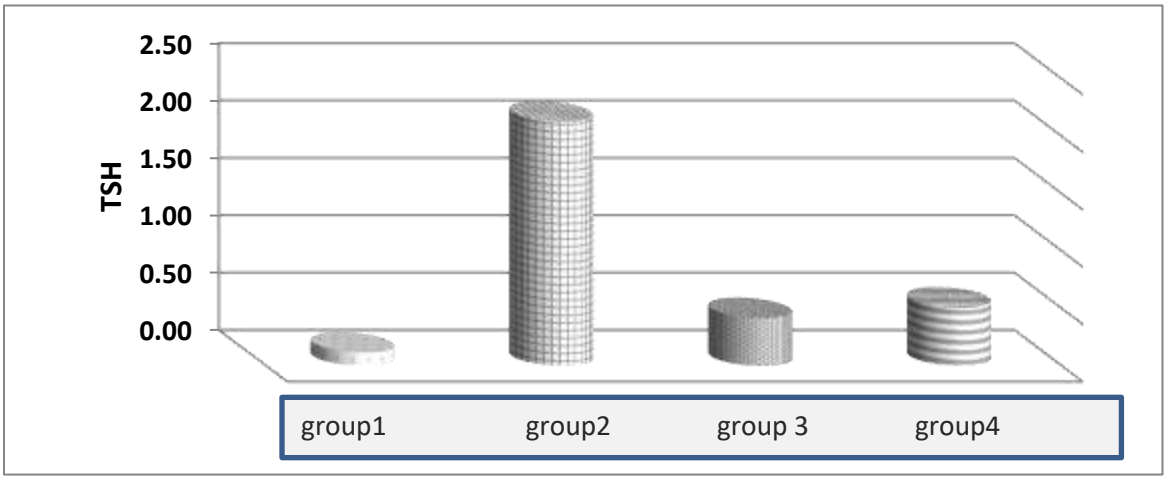

Figure 1: the effect of aqueous extract (group 3) and oil (group 4) on TSH compared to control (group 1) and Hypothyroidism group 2.

Comparison of the means value of FT3 in the studied group:

Table (1) and scheme (2) show that the means value of FT3 has significantly decreased $p<0.05$ after inducing the hypothyroidism to increase significantly $p<0.05$ after treatment with extract and oil.

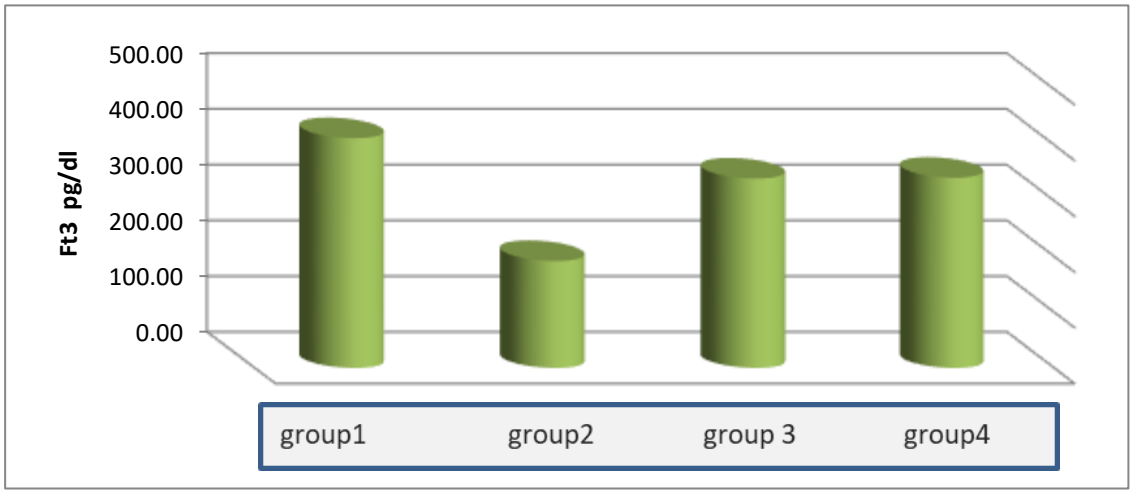

Figure 2: the effect of aqueous extract (group 3) and oil (group 4) on FT3 compared to control (group 1) and Hypothyroidism group 2.

Comparison of the means value of FT4 in the studied group:

Table (1) and scheme (3) show that the means value of FT4 has significantly decrease $p<0.05$ after induced the hypothyroidism to insignificantly increased $p>0.05$ after treating with extract, while it was significantly increased $p<0.05$ after treating with oil.

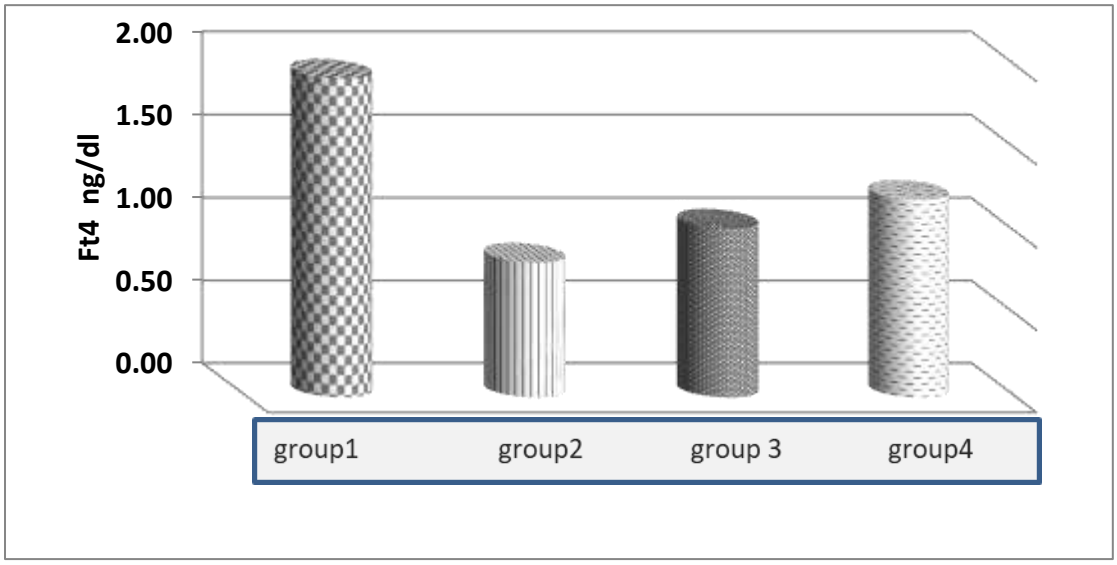

Figure 3: clarifies the effect of aqueous extract (group 3) and oil (group 4) on FT4 compared to control (group 1) and Hypothyroidism group 2.

DISCUSSION

The orally administered white mice a dose of 0.05 mg/kg/body weight /day for 3 weeks caused significant deceasing (P<0.05)in hormone level (FT4,FT3), it also caused significant increasing (P<0.05) in TSH level hormone, that is a result of induced the hypothyroidism in these mice, where methimazole acts as a false Iodide for thyroid peroxidase, thus blocking the iodination of thyrosine residues within thyroglobulin, subsequently occurring decreasing its hormones levels in blood serum[23], this stimulates the anterior lobe of the pituitary gland to increase TSH secretion to retrieve this decreasing, in sense that the thyroid gland is under control of the regulation of the hypothalamic-pituitary-thyroid axis (Feedback Negative), and this explains the level height of TSH after orally methimazole[24].

As well as after orally administered of aqueous extract of *N. sativa* and its oil, significant increasing p<0.05 happened in FT3 levels, and significant decrease p<0.05 in TSH levels, also significant increasing<0.05 happened in FT4 levels when Nigella Sativa oil was dosed, but this increasing was un significant p>0.05 in the level of FT4 when Aqueous extract of Nigella Sativa was dosed.

The results of this study had agreed with Ebrahim *et al* it had shown that oral administration of thyroid healthy male rats with *N. sativa* alcoholic extract at doses (25,50,100)mg/kg/weight body/day for a month causes a significant increasing p<0.05 in T3 level, and insignificant in T4 level, that because the *N. sativa* contents of amino acids specially tyrosine which consider the essential substrate to make the thyroid hormone, besides antioxidant that improve the functioning of thyroid gland [25]. It also agreed with Abou- Zena et al were adding *N. sativa* powder 2% to fodder which feed goats' kids to significantly increasing in T3 and insignificantly increasing in T4, also the researcher found semi-results when adding supplement which contents (Vit E, Zn) [26]. And Sharif et al. 2012 who pointed to Orally treatment by *N. sativa* ethanol extract with a daily 1g/kg caused a significant increasing in thyroid hormones levels and the cause of this large increasing in T3 level is activity increasing enzyme deodenase-5 which works on

transforming T4 to T3. As well as agreed with Khalawi et al (2013) which clarified that 400mg/kg dose of Nigella Sativa oil for a month had changed PTU induced hypothyroidism statue to hyperthyroidism [27].

Nigella Sativa contains thymoquinone which plays an important role in thyroid hormones biosynthesis, because of this compound ability to increase thyroid hormone production and deletion oxidative stress accompanying to hypothyroidism [28] ,this compound is soluble in oils (Fat) [29]which makes NSO superior to aqueous extract in improving FT4 level, as well as Nigella Sativa oils contain triterpene saponin and many other terpenes and phenolic compounds, which play roles as antioxidants to[30,31].And polyphenols compounds as α,β,γ Tocopherol (Pro-Vit E) that many functions in increasing thyroid hormones levels [32]. In addition to fatty acids function especially linoleic acid (Omega 3) that regulate thyroid hormones secretion and antioxidant function[33, 34] .

In this context NSE and its oil contain many important minerals to thyroid gland function besides the antioxidant role such as Zn and Cu[35] . selenium which is considered accompanying to deodenas-5 enzyme and additionally, it's a part of glutathione peroxides(one of the most important antioxidants in thyroid gland)[36].

The result of this study had also agreed with many studied of medical plants to treat hypothyroidism. Where *Ginge*r extract has a role in the treatment of hypothyroidism albino mice, it caused significant increasing $p<0.05$ in T3, T4 levels, and significant decreasing $p<0.05$ in TSH level [37] . And phenolic extract of convolvulus arvensis had related effect to the levothyroxine efficiency in treating the hypothyroidism [38] .And agreed with Abu-Fotouh et al that shows the effect of curcumin extract with 100mg/kg dose in treating hypothyroidism induced by potassium dichromate in white mice , because curcumin contains polyphenol yellow dye which function as a free radicals remover [39] . It also agreed with Osman et al which referred to the aqueous extract ability with 10% concentration in treating the induced hypothyroidism in white mice, this goes back to content of the extract of antioxidants for example thymol and other phenols [40, 41] .

Statistical analysis

The results were statistically analyzed using the social package for social sciences program (SPSS). A on way test of variance (ANOVA) was conducted to determine if there were significant differences among the studied groups, then the LSD5% test was used to located these differences, and the result of LSD test are shown in a letter method, where the means are arranged in ascending order, then every two groups have a common letter, the difference between them is not significant (the difference between them is less than the LSD value)

CONCLUSION

We studied the efficacy of aqueous *Nigella sativa* extract and its oil in treating hypothyroidism induced methimazole in white mice and both of them had effectiveness in increasing FT3 level and decreasing TSH level, but *Nigella Sativa* oil had larger effect in increasing FT4 level (in sense that increasing was significant $p<0.05$) and it was not significant in FT4 level when using aqueous extract.

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مقارنة بين تأثير المستخلص المائي للحبة السوداء وزيتها على قصور الدرق المستحدث بالميثمازول لدى الفئران البيضاء

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ملخص

الهدف: أجريت هذه الدراسة بهدف تقييم تأثير كل من المستخلص المائي للحبة السوداء وزيتها في علاج قصور الدرق المستحدث بالميثمازول لدى الفئران البيضاء بهدف الوصول إلى علاج طبيعي لقصور الدرق بدلاً من العلاجات الكيميائية ذات التأثيرات الجانبية الخطيرة.

المواد المستخدمة والطرق: شملت الدراسة 40 من إناث الفئران البيضاء البالغة، وزعت على أربع مجموعات، المجموعة 1 (شاهدة فيزيولوجية): جرعت بمحلول فيزيولوجي 0.09 طيلة فترة التجربة. المجموعة2 (شاهدة مرضية) جرعت بعقار الميثمازول بتركيز 0.05 ملغ/كغ يومياً لمدة 3 أسابيع لاستحداث القصور الدرقي. المجموعة 3: استحدث فها قصور الدرق ثم عولجت بمستخلص الحبة السوداء المائي بجرعة 400 ملغ/كغ لمدة شهر. المجموعة 4: استحدث فيهل قصور الدرق ثم عولجت بزيت الحبة السوداء بجرعة 1كل/كغ.

النتائج: بينت النتائج أن كلا المستخلص والزيت سبب انخفاضاً معنوياً$P<0.05$ في مستو هرمون TSH، وارتفاعاً معنوياً $P<0,05$ في مستو هرمون FT3 ، وسبب زيت الحبة السوداء ارتفاعاً معنوياً في مستوى هرمون FT4 بينما لم يكن الارتفاع معنوياً عند استخدام المستخلص المائي للحبة السوداء.

الكلمات الدالة: زيت الحبة السوداء، المستخلص المائي للحبة السوداء، FT3، FT4، TSH.

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Osmotic Stress Enhances Antimicrobial Activity of in Vitro Grown Microshoots of *Ochradenus Baccatus* Delile Against Selected microbes

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ABSTRACT

Ochradenus baccatus Delile is a wild medicinal plant that produces several natural compounds with medicinal benefits. In this study, microshoots of *Ochradenus baccatus* were exposed to osmotic stress conditions consisting of Murashige and Skoog solid media (MS) media that containing different osmotic agents (sugar types) at a range of concentrations (including 0.1, 0.2, 0.3 or 0.4 M). The purpose was to test their effects on microshoots' growth and antimicrobial activities against selected strains of bacteria and one strain of fungi. It has been found that growth parameters (including shoot length and proliferation) of *Ochradenus baccatus* microshoots declined with increasing sugar concentration in the media, but at the highest concentrations of 0.4M mannitol, the micorshoots could not survive. Generally, aqueous extracts of the stressed microshoots were more effective against the tested microbial strains than the methanolic extracts in most experiments. *Staphylococcus aureus* was found to be the most affected microbe to both extract types. Also, exposing the microshoots to osmotic stress had improved antimicrobial powers in both extracts types. Aqueous extract of microshoots that pre-grew in media with (0.4 M sucrose) was interestingly found to inhibit growth of *Staphylococcus aureus* and *Candida albicans* with minimal inhibitory concentration (MIC) values of (0.195, 0.78 mg/ml). These values were similar to those obtained from the antibiotic treatments. Other biotechnological techniques like genetic transformation are suggested to be also used for production of elite strains of *Ochradenus baccatus* with super antimicrobial potential.

Keywords: Antimicrobial activity; *in vitro*; microshoots; *Ochradenus baccatus*; Osmotic agent, MIC, mannitol, sucrose.

1. INTRODUCTION

For ages, plants comprised a source for defense and prevention of disease [1-6]. Interestingly, millions of people consider plant medicine as the main source of health care[7]. Recently, synthetic antibiotics were reported to have a close relation to the high mortality rates among people due to the harmful outcomes of overuse and misuse of antimicrobial treatments on vital body organs and cells, as well as adverse effect on the immune system [8-10].

To find a solution there is a need to find alternatives to these chemical antibiotics. However, establishment of robust and suitable methods to provide continuous and effective alternatives against microbes might be provided

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by tissue culture techniques, which are independent on external environmental conditions [11-14]. *Ochradenus baccatus* Delile is a member of *Reseadasea* family [15] and widely spread in Middle Eastern deserts from Pakistan to the north of Africa [16]. In Jordan, *O. Baccatus* presents in Jordan valley, Dead Sea area, Wadi Araba, Al Karak and Wadi Mosa. This herb was reported to possess antibacterial, hepatoprotective, anti-inflammatory, anticancer, and antiviral activities which refer to the presence of several natural compounds such, as phenols and flavonoids [17]. *O. baccatus* is extensively used by the locals as a treatment of inflammations, high blood cholesterol and sexual disorders [18-19]. Unfortunately, uncontrolled collection of *Ochradenus baccatus* Delilah, is exposing this valuable herb to extinction. Finding a method that enables rapid and massive production of *Ochradenus baccatus* Delile with improved antimicrobial powers would contribute highly to rescue this plant degradation, and help to obtain a sustainable source of microshoots with elite curing powers without jeopardizing the plants grown in the wild.

Tissue culture techniques offer chances for production of effective compounds from the plant without depending on the wild [20], by applying techniques that allow rapid and massive production of microshoots which would consequently supply the target natural compounds *in vitro* [21]. This study was conducted to investigate the effect of osmotic stress on antimicrobial powers of two extract types collected from *Ochradenus baccatus in vitro* grown microshoots against different microbes, and to compare the results with those obtained from the wild type plant and the antibiotic.

2. MATERIALS AND METHODS:

2.1 *In vitro* establishment of mother stock of *Ochradenus baccatus*

In the materials section; all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as mentioned specifically. Seeds of *Ochradenus baccatus* were sterilized by washing under tap water for 20 minutes then seeds were immersed in 4.0% sodium hypochlorite plus two drops of Tween–20 for 15 minutes. After that, the seeds were rinsed with autoclaved distilled water three times, then the seeds were soaked in 70% (v/v) ethanol solution for 30 seconds before being rinsed with sterile distilled [22].

The sterilized seeds were inoculated on the surface of petri dishes containing either of MS (1962) [23] solid media supplemented with 2.0 mg/L Gibberellic acid (GA_3), or hormone free MS media. Then the plates were kept at 21± 2°C under two light regimes consisted of complete darkness, or daily light regime in growth room conditions; of 16-h (photosynthetic photon flux density (PPFD) = 40 µmol. m^{-2} sec^{-1}) for two weeks. The germinated parts that developed cotyledonary stage were subcultured into 250 ml Erlenmeyer flasks (100 ml media / flask) containing hormone-free MS medium under daily light regime growth room conditions for four weeks.

After one month of seed germination nodal segments (1.0 cm) were subcultured into MS media supplemented with 1.0 mg/L of benzyl adenine (BA) [24] under completely sterile conditions. Cultures were incubated in growth room under daily light regime of 16-h (photosynthetic photon flux density (PPFD) = 40 µmol. m-2 sec-1) light, 8-h dark at 24±1°C. Microshoots were subcultured every four weeks and kept under the same mentioned conditions to initiate enough microshoots.

2.2 Studying the effect of different types and concentrations of osmotic agents on microshoots growth of *Ochradenus baccatus*

Microshoots were subcultured onto MS medium without growth regulators for 3-4 days to eliminate the carry-over effects of growth regulator. Then, 1.0 cm length of *Ochradenus baccatus* microshoot were subcultured into half strength (HS) MS medium supplemented with (0.1, 0.2, 0.3 or 0.4 M) of either sucrose, sorbitol or mannitol of 20 replicates of each treatment, the data were collected after 8 weeks of culture for microshoots length and number of microshoots, then samples were dried and kept till use for extraction.

2.3 Assessment of antimicrobial activity of wild and *in vitro* grown microshoots

2.3.1 Plant extracts preparation

Samples of wild type and in *vitro* grown microshoots of *Ochradenus baccatus* were dried in the shade for two weeks, and then grounded into fine powder by using a blender. The wild type samples (shoots and leaves) were collected from 31° 59 6 223 N, 35° 59 00 73 E (AL-Zarah) Dead Sea area, May 2016.

For aqueous extract, 8.0 g of the dried plant powder were taken from samples of wild type in addition to the in vitro grown microshoots (grown either under stressing or normal in vitro growth conditions) and were mixed with 50 ml autoclaved distilled water in clean bottles for 24 h at 24°C with shaking using incubator shaker (Sheldon Manufacturing, Inc.®; Cornelius, OR USA), then centrifuged at 2000 rpm for 10 min, before the resulted supernatant was collected [25]. After that, the water was evaporated by a rotary vacuum evaporator (RE300 rotary evaporator; Stuart Vacuum Pump - RE3022C, Staffordshire, UK); [26]. The residue was weighed, then dissolved in dimethyl sulfoxide (DMSO) [27] to obtain 100 mg/ml of stock extract then stored in refrigerator.

Methanolic extract was prepared by stirring 1 g of dried plant powder of the wild plant, *in vitro* grown microshoots (osmotic stress, non- stressed) in 10 ml of 100% methanol for 4h [25], followed by centrifugation at 2000 rpm for 10 minutes. After evaporating the methanol by rotary vacuum evaporator, the supernatant was dissolved and stored in DMSO at a concentration of 100 mg/ml as stock extract and stored in refrigerator [26]. Extracts from all these preparations were tested for purity by plotting them on Mueller Hinton Agar (MHA) and incubated for 24 hours at 37°C.

2.3.2. Evaluating antimicrobial activity

Five pathogenic microbes were selected for this study. Of these microbes are Gram-negative bacteria (e.g., *Escherichia coli* ATCC (8739) and *Klebsiella pneumonia* ATCC (31488)) and Gram-positive bacteria (e.g., *Staphylococcus aureus* ATCC (6538) and *Bacillus subtilis* ATCC (6633)), and one species of Fungi called *Candida albicans* purchased from ATCC (10231). These strains were supplemented by the microbiology laboratory at Hamdi Mango Center for Scientific Research.

2.3.2.1 *Inoculum Standardization*

Bacterial – fungal aliquots were prepared by dissolving one well - isolated bacterial colony into 3 ml of 0.9 % NaCl, while isolated fungi into 3 ml of sterilized distilled water. Then, the turbidity adjusted to 0.5 McFarland standard, to obtain aliquots containing approximately 1x10^8 CFU/ml.

2.3.2.2 *Bacterial and fungal broth Preparation*

Bacterial and fungal broth at 1:100 dilution was prepared by transferring 10 µl of previous prepared aliquots to 1.0 ml of Muller Hinton broth for bacteria, and to 1.0 ml of potato dextrose broth for fungi in order to carry out antimicrobial assay.

2.3.2.3 *Antimicrobial assay (Broth Microdilution)*

Different *O. baccatus* extracts; wild and *in vitro* grown microshoots (osmotic stress, non-stressed) were used to study their antimicrobial activity. MIC was determined by broth microdilution method, using 96-wells microplates. To do this, 50µl of Muller Hinton broth were added to each well of the first row (well A1) then serially diluted from (A1 to A10) by taking 50 µl of each well on the same row making a two-fold serial dilution ranging from concentration 25 to 0.09 mg/ml of extract, the last 50µl were discarded. Then, the ten wells were inoculated with 50µl of previously prepared bacterial or fungal broth.

Well number eleven in each row consisted of nutrient broth plus Tetracycline (10 mg/mL) and was used as a control. Testing50µl of DMSO instead of the extract was carried out to confirm the lack of interference of DMSO. The plates were covered and incubated at 37ºC for 24 hr. After the incubation period, the plates were scanned with an ELISA reader (BioTek® 800™ TS Absorbance Reader instrument, USA) at 600 nm [27] for bacteria, and at 405 nm for fungi [28]. The lowest concentration of extract that prevent microbial growth represented MIC [29]. Each

experiment in section 2.3 (Assessment of antimicrobial activity of wild and in vitro grown microshoots) was repeated three times with three replications.

2.4 Experimental design

Treatments of the experiment: (Effect of different types and levels of osmotic agents on microshoots growth of *Ochradenus baccatus*) were arranged in a completely randomized design (CRD). Each treatment consisted of twenty replicates (test tube) each with one microshoot to find out the effect of osmotic agent type and level on growth parameters of the microshoots (microshoot length and number of new proliferated shoots). The collected data were statistically analyzed using SPSS analysis system. Analysis of variance (ANOVA) was used and means separation was performed at probability level of 0.05 according to the Tukey's HSD. Antimicrobial experiments were repeated three times, and as mean values collected from antimicrobial experiments were discrete values (observed (+), not observed (-) they didn't undergo statistical analysis.

3.0 RESULTS AND DISCUSSION

3.1 Effects of different osmotic agents on *in vitro* growth of microshoots

Growth responses of *O. baccatus* microshoots varied according to the osmotic agent type and concentrations. Sucrose at the concentration of 0.1 M (the control) was obviously the best one for the growth of *O. baccatus* microshoots, as shoot length reached (5.4 cm) and 4.5 new microshoots developed (as seen in Table 1). However, increasing sucrose concentrations resulted in a clear reduction in the growth parameters to reach the minimum level at 0.4 M concentration of sucrose (as found in Table 1 and Figure 1). Our data were in agreement with results reported by studies [30- 32] who found that the growth and proliferation of *Achilliae fragrantissima, Thymbra spicata* and *Teucrium polium* microshoots decreased when grown under similar concentrations of sucrose. Similar trend of reduction in the growth parameters of microshoots when they exposed to similar range of concentrations of sorbitol and mannitol. However, the growth reduction was more severe in microshoots grown in 0.4 M concentration of mannitol comparing to other concentrations and other types of sugars with no survival microshoots.(Table 1). The continuous exposure of mannitol was reported to be toxic to the plant cells of *Oryza sativa* L. and *Ruta graveolens*. High concentration of mannitol decreased water uptake, decreased cell division, increased electrolyte leakage in the microshoots [33-34]. It is commonly known that adding high levels of sugars to the culture media would restrict water availability to plant cells and expose them to osmotic stress, which would force the cells to minimize their division as a defense mechanism [35].

Table 1: Effects of osmotic agent type and level on *in vitro* growth of *Ochradenus baccatus* microshoots after incubation for (8 weeks)

Concentration (M)	**Microshoots length (cm)**		
	Sucrose	**Sorbitol**	**Mannitol**
0.1 (control)	5.4 ± 0.31	4.1 ± 0.05	3.3 ± 0.07
0.2	4.1 ± 0.15	3.5 ± 0.05	2.2 ± 0.10
0.3	2.3 ± 0.02	1.7 ± 0.04	1.2 ± 0.07
0.4	1.1 ± 0.01	1.4 ± 0.05	-
	Number of proliferated shoots		
	Sorbitol	**Sucrose**	**Mannitol**
0.1	3.3 ± 0.41	4.5 ± 0.05	2.16 ± 0.07
0.2	2.1 ± 0.15	3.6 ± 0.25	1.2 ± 0.13

Concentration (M)	Microshoots length (cm)		
0.3	1.3 ± 0.02	2.8 ± 0.14	1.0 ± 0.07
0.4	1.1 ± 0.08	2.1 ± 0.05	-

(-): microshoots died after 8 weeks of incubation in this treatment

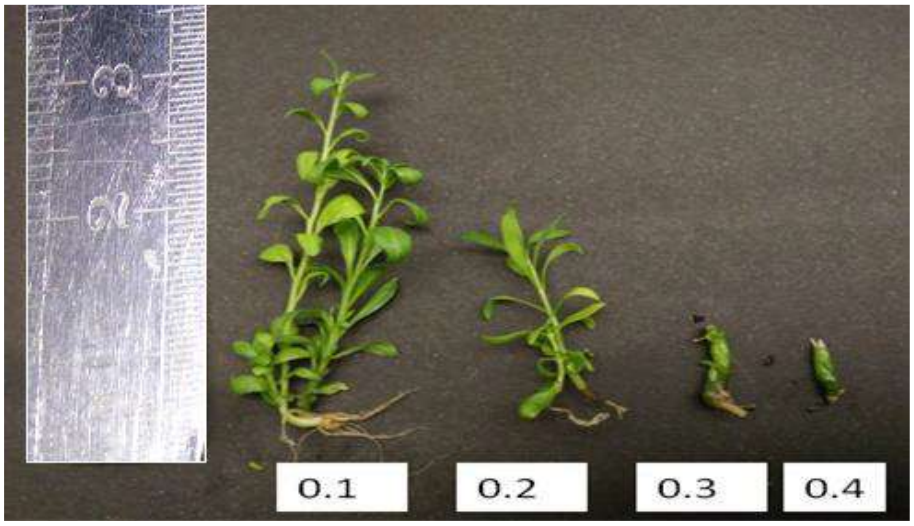

Figure 1: Microshoots length on MS media supplemented with (0.1, 0.2, 0.3, 0.4) M sucrose after 8 weeks.

3.2 Assessment of antimicrobial activity

3.2.1 ***Antimicrobial activity of in vitro grown Ochradenus baccatus microshoots under different concentrations of sucrose***

Antimicrobial activity was detected in both methanolic and aqueous extracts of *Ochradenus baccatus*. Interestingly, extracts collected from the microshoots pregrown in growth media contained increased sucrose levels have shown increasing antimicrobial powers of both extracts, although growth parameters of microshoot were decreased (Tables 1, 2). For example, antimicrobial potential against *Staphylococcus aureus* and *Candida albicans* was highly improved in methanolic extracts of *Ochradenus baccatus* microshoots grown in media with (0.4 M sucrose) with MIC values of 0.39 and 3.12 mg/ml, respectively. These MIC were similar to results obtained from the wild-type plant and the antibiotic with MIC values of 0.195, 0.78 mg/ m) (Table 2).

Adding high levels of sucrose to the culture media was reported to improve the production of secondary metabolites in cell and organ cultures for many plant species [36-39] and consequently improve antimicrobial powers of the resulted extract although growth of microshoots was adversely affected [40]. For example, cell culture of *Ginkgo biloba* showed maximum growth at 3% sucrose, but significant decline in the cell biomass at higher levels of sucrose of 5 and 7%. Interestingly, the aforementioned concentrations were the best for production of active ingredients (such as ginkgolides and bilobalides) [40].

Moreover, our data revealed that *Staphylococcus aureus* was the most affected microbe to both extract types of the microshoots., it was clear from the results that aqueous extract of the microshoots and the wild-type plant had stronger inhibition powers against all tested microbes compared to methanolic extracts (Table 2). This indicated that antimicrobial powers of the microshoots of *O. baccatus* varied with sucrose concentrations and extract type. Our findings were comparable with that obtained by [25] study who

found, that activity of *O. baccatus* aqueous extracts against nematode were more effective than methanolic extracts. It is worth mentioning that antimicrobial properties of *O. baccatus* were attributed to active ingredients produced after glucosinolate hydrolysis by enzyme myrosinase. The hydrolysis only happens in the presence of water and moderate temperature [41], which might explain the superiority of aqueous extracts over methanolic extracts.

On the other hand, study [19] found that other extract types (ethanolic and n-hexane extracts) of *O. baccatus* wild type plants were more efficient than aqueous and methanolic extracts at killing microbes, as ethanolic extract was effective against *Staphylococcus aureus; Escherichia coli*; and the fungus, *Candida albicans*, whereas n-hexane extracts was effective against *Candida albicans.*

Table 2. Effect of different concentrations of sucrose on antimicrobial activity of methanolic and aqueous extracts collected from *in vitro* grown *Ochradenus baccatus* microshoots growing under osmotic stressing conditions

Methanolic extract						
Sucrose concentration (M)						
Treatment	**0.1**	**0.2**	**0.3**	**0.4**	**Wild-type plant**	**Tetracycline (control)**
Staphylococcus aureus	3.12	1.56	0.78	0.39	0.39	0.195
E. coli	-	6.24	6.24	3.12	3.12	0.39
Klebsiella pneumoniae	6.24	6.24	3.12	3.12	1.56	0.78
Bacillus subtilis	3.12	3.12	1.56	1.56	0.78	0.195
Candida albicans	6.24	6.24	3.12	3.12	3.12	0.78
Aqueous extract						
Sucrose concentration (M)						
Treatment	0.1	0.2	0.3	0.4	Wild type plant	Tetracycline (control)
Staphylococcus aureus	1.56	0.78	0.39	0.195	0.195	0.195
E. coli	6.24	3.12	1.56	1.56	0.78	0.39
Klebsiella pneumoniae	6.24	6.24	3.12	1.56	0.78	0.78
Bacillus subtilis	1.56	1.56	1.56	0.78	0.39	0.195
Candida albicans	3.12	3.12	1.56	0.78	0.78	0.78

* Values represent the means of minimal inhibitory concentrations MIC (mg/ml) of the extract needed to inhibit growth of each microbe. (-): No inhibition observed.

3.2.2 ***Antimicrobial activity of in vitro grown Ochradenus baccatus microshoots under different concentrations of sorbitol***

Antimicrobial powers of the microshoots increased in microshoots pregrwon in MS media supplemented with higher concentrations of sorbitol levels (Table 3). Both extract types from the microshoots showed antimicrobial activities against most tested microbes except *Candida albicans* (Table 3). On the other hand, growth of *Candida albicans* was inhibited upon treatment with aqueous and methanolic extracts of the wild-type plant with MIC values of 3.12 and 0.78 mg/ml, respectively (Table 3). *Staphylococcus aureus* was again the most affected microbe when exposed to sorbitol treatment in both extract types (Table 3). Additionally, aqueous extract kept showing better performance against all microbes than methanolic extract (Table 3).

Table 3. Effect of different concentrations of sorbitol on antimicrobial activity of methanolic and aqueous extracts collected from in vitro grown *Ochradenus baccatus* microshoots growing under osmotic stressing conditions

Methanolic extract						
Sorbitol concentration (M)						
Treatment	**0.1**	**0.2**	**0.3**	**0.4**	**Wild type plant**	**Tetracycline (control)**
Staphylococcus aureus	3.12	1.56	0.78	0.78	0.39	0.195
E. coli	-	-	12.48	6.24	3.12	0.39
Klebsiella pneumoniae	12.48	6.24	3.12	3.12	1.56	0.78
Bacillus subtilis	3.12	3.12	1.56	1.56	0.78	0.195
Candida albicans	-	-	-	-	3.12	0.78
Aqueous extract						
Sorbitol concentration (M)						
Treatment	0.1	0.2	0.3	0.4	Wild type plant	Tetracycline (control)
Staphylococcus aureus	3.12	1.56	0.39	0.39	0.195	0.195
E. coli	-	6.24	3.12	3.12	0.78	0.39
Klebsiella pneumoniae	-	6.24	1.56	1.56	0.78	0.78
Bacillus subtilis	3.12	3.12	1.56	0.78	0.39	0.195
Candida albicans	-	-	-	-	0.78	0.78

* Values represent the means of minimal inhibitory concentrations MIC (mg/ml) of the extract needed to inhibit growth of each microbe. (-): No inhibition observed.

3.2.3 ***Antimicrobial activity of in vitro grown Ochradenus baccatus microshoots under different concentrations of mannitol***

Aqueous and methanolic extracts of *Ochradenus baccatus* microshoots were observed to have antimicrobial action against three types of bacteria including *Staphylococcus aureus, Klebsiella pneumoniae* and *Bacillus subtilis upon exposure to mannitol (*as seen in Table 4*).* This was exclusively noticed in extracts collected from microshoots pregrown with increasing concentrations of mannitol, with the exception for the highest concentration of 0.4M mannitol because microshoots could not survive after 8 weeks of incubation at this concentration (Table 4). Also, it was clear from Table 4 that growth of *E. coli* was inhibited when exposed to aqueous and methanolic extract of the wild-type plant with MIC of (12.48 and 3.12 mg/ml, respectively), but it wasn't affected by any extract type collected from the microshoots. Meanwhile, *Candida albicans* wasn't affected by any extract type of *O. baccatus* (as seen in Table 4).

Many stress physiology research articles have shown that adding high concentrations of sugars was found to improve secondary metabolites production in *in vitro* grown plants [37-38; 42]. This might explain that improvement in antimicrobial activity of both extracts of microshoots in response to the addition of high sugar concentrations.

Table 4. Effect of different concentrations of mannitol on antimicrobial activity of methanolic and aqueous extracts collected from in vitro grown *Ochradenus baccatus* microshoots growing under osmotic stressing conditions

Methanolic extract					
Mannitol concentration (M)					
Treatment	0.1	0.2	0.3	Wild type plant	Tetracycline (control)
Staphylococcus aureus	3.12	3.12	1.56	0.39	0.195
E. coli	-	-	-	12.48	0.39
Klebsiella pneumoniae	6.24	6.24	3.12	1.56	0.78
Bacillus subtilis	6.24	6.24	1.56	0.78	0.195
Candida albicans	-	-	-	-	0.78
Aqueous extract					
Mannitol concentration (M)					
Treatment	0.1	0.2	0.3	Wild type plant	Tetracycline (control)
Staphylococcus aureus	3.12	1.56	0.39	0.195	0.195
E. coli	-	-	-	3.12	0.39
Klebsiella pneumoniae	6.24	6.24	3.12	0.78	0.78
Bacillus subtilis	3.12	1.56	1.56	0.39	0.195
Candida albicans	-	-	-	-	0.78

* Values represent the means of minimal inhibitory concentrations MIC (mg/ml) of the extract needed to inhibit growth of each microbe. (-): No inhibition observed.

4. CONCLUSIONS

It can be concluded from the obtained data that growth responses of *Ochradenus baccatus* microshoots varied with osmotic agent type and level. Adding 0.1M sucrose to the media resulted in the best growth for the microshoots, while adding higher levels of all sugar types affected microshoots growth adversely.

Meanwhile, antimicrobial activity of *Ochradenus baccatus* was found to be enhanced in extracts collected from microshoots pregrown under osmostressing conditions. The reason behind this can be due to fact that plant cells tend to produce more active ingredients including secondary metabolites to balance cell water potential in order to reduce water loss resulted from cells exposure to high osmotic stressing conditions. However, our results showed that the best antimicrobial results were recorded by extracts collected from microshoots samples pregrown in vitro in media contained either (0.3 or 0.4) M of sucrose or sorbitol.

More research is needed on the extract of *Ochradenus baccatus* to find out exactly the types of secondary metabolite that are responsible for the antimicrobial powers of this plant and how other tissue culture techniques can be applied to improve production of these compounds.

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6. Conflict Of Interest:

The authors can hereby confirm that they have no conflict of interest in this manuscript.

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الإجهاد الاسموزي يعزز النشاط المضاد للميكروبات لنبات الأرضه *Ochradenus Baccatus* Delile المزروع داخل الانابيب في المختبر ضد ميكروبات مختاره

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ملخص

نبات الارضة *Ochradenus baccatus* Delile هو نبات طبي بري ينتج العديد من المركبات الطبيعية ذات الفوائد الطبية. في هذه الدراسة، تم تعريض الفروع الصغيرة من نبات الارضه *Ochradenus baccatus* داخل الانابيب الى ظروف الإجهاد الاسموزي و التي تتكون من وسائط غذائية صلبة موراشيج و سكوج Murashige و Skoog (MS) و التي تحتوي على عوامل أسموزية مختلفة (أنواع سكريات) في مدى من التركيزات (بما في ذلك 0.1 ، 0.2 ، 0.3 أو 0.4 مولار) . كان الهدف هو اختبار تأثير هذه السكريات على نمو السيقان الدقيقة للنبات داخل الانابيب والأنشطة المضادة للميكروبات لهذا النبات ضد سلالات مختارة من البكتيريا وسلالة واحدة من الفطريات. لقد وجدنا أن عوامل النمو (بما في ذلك طول النبتة وتكاثرها) من سيقان نبات الارضه داخل الانابيب *Ochradenus baccatus* انخفضت مع زيادة تركيز السكر في الوسط الغذائي ، ولكن عند أعلى تركيز 0.4 مولار مانيتول، لم تتمكن السويقات الدقيقة داخل الانابيب من البقاء. بشكل عام ، كانت المستخلصات المائية للسويقات الدقيقة للنبات داخل الانابيب المعرضه للإجهاد الاسموزي أكثر فعالية ضد السلالات الميكروبية المختبرة من المستخلصات الميثانولية في معظم التجارب. تم العثور على المكورات العنقودية الذهبية *Staphylococcus aureus* لتكون الميكروب الأكثر تضررا من كلا النوعين من المستخلصات. أيضًا ، أدى تعريض البراعم الدقيقة للإجهاد الاسموزي إلى تحسين القوى المضادة للميكروبات في كلا النوعين من المستخلصات. المثير للاهتمام أن المستخلص المائي السيقان الدقيقة للنبات داخل الانابيب التي نمت مسبقًا في الوسط الغذائي باستخدام (0.4 مولار سكروز) عملت على تثبيط نمو المكورات العنقودية الذهبية *Staphylococcus aureus* والخمائر البيضاء *Candida albicans* باستخدام طريقة التثبيط عند التركيز الاقل (MIC) بقيم (0.195 ، 0.78 مغم / مل). كانت هذه القيم مماثلة لتلك التي تم الحصول عليها من العلاجات بالمضادات الحيوية. يُقترح أيضًا استخدام تقنيات التكنولوجيا الحيوية الأخرى مثل التحول الجيني لإنتاج سلالات النخبة من *Ochradenus baccatus* مع إمكانات فائقة لمضادات الميكروبات.

الكلمات الدالة: نشاط مضاد للميكروبات، داخل الانابيب، السويقات، نبات الارضه، عامل أسموزي، التثبيط عند التركيز الاقل (MIC)، سكر مانيتول، سكر سكروز.

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Valerian and Hops Combination Versus Escitalopram in Models of Depression and Anxiety: A Cross-talk with Oxidative Stress

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ABSTRACT

Depression and anxiety disorders are the most common mental health problems and are associated with oxidative stress. Although famous for its anxiolytic effect, the antidepressant effect of the valerian-hops combination was not previously studied, also the relationship between the sedative effect of valerian-hops and oxidative stress markers is unclear. The current research has two objectives: (1) to compare the antidepressant effect of valerian-hops with escitalopram and (2) to evaluate the sedative/anxiolytic effects of valerian-hops in relation to oxidative stress markers namely Nitric Oxide (NOx), inducible Nitric Oxide Synthase (iNOS) and Super Oxide Dismutase (SOD). Two models were employed using BALB/c mice: A normal condition depression model in which mice were divided into: control, valerian-hops-treated (100mg/kg), and escitalopram-treated (10mg/kg) groups one hour before the open field test, the elevated plus-maze test, and the forced swim test and an anxiety model in which mice were divided into: unstressed naïve, control (stressed), valerian/hops (100mg/kg), and escitalopram (10 mg/kg) groups treated for three weeks; acutely restrained for 6 hours and sacrificed, serum was obtained to detect NOx, iNOS and SOD activity. In the depression model, valerian-hops demonstrated antidepressant activity similar to escitalopram ($p>0.05$). In the anxiety model, the valerian-hops treated mice demonstrated a profound sedative effect in all behavior paradigms ($p<0.05$), and normalized the anxiety-induced NOx levels and SOD activity ($p<0.05$). Under normal conditions, the valerian-hops combination exerts an antidepressant effect similar to escitalopram while in stress/anxiety conditions it exerts profound sedative and antioxidant effects.

Keywords: Antidepressants, anxiolytics, valerian-hops, mice model, stress.

1. INTRODUCTION

Mental health disorders are becoming increasingly common among all age groups (1,2) Depression and anxiety disorders are the most common mental health problems and are dramatically increasing worldwide (3). The neurobiology of depression and anxiety is complex and multifactorial. Beyond the well-established monoamine deficiency theory of psychological disorders (4), accumulating evidence points to the role of oxidative stress(5,6). Nitrates, a nitric oxide (NO) metabolite, are a marker of oxidative stress and were shown to increase during acute anxiety (7,8). Nitric oxide (NO) is produced from L-arginine by enzymatic conversion of the enzyme NO synthase (NOS) (9). In support of the potential role of NO in depression, a growing body of evidence has demonstrated that some antidepressants exert a NO-lowering effect. For example, a study revealed that L-arginine antagonized the effects of the classic tricyclic

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antidepressant imipramine (10). Superoxide dismutase (SOD) is an abundant antioxidant enzyme responsible for superoxide (O2-) species detoxification to yield hydrogen peroxide (H_2O_2). SOD activation occurs as a response to an oxidative stress status (11).

The different classes of available antidepressants are based on the monoaminergic deficiency hypothesis, i.e. they increase the levels of the synaptic monoamines (serotonin, dopamine, and norepinephrine); these classes include Selective Serotonin Reuptake Inhibitors (SSRIs) such as escitalopram. Although considered a safe option, SSRIs have many limitations: they are effective only in 50% of cases, they could lead to weight gain or sexual impairment, and they take up to two weeks to exert a clinically acceptable effect (12,13). Therefore, herbs with psychotropic effects are gaining wide attention due to their high safety profile, minimal drug interactions, and negligible addictive potential (14–16).

Valerian and hops are perennial plants. valerian is originally native to Asia and Europe (17) and hops plants are distributed throughout North America, Europe, and Asia (18). Valerian root (*Valeriana officinalis Valerianacea*) is often combined with Hops strobile (*Humulus lupulus L., Cannabaceae*) to enhance its sedative and anxiolytic efficacy (19). The pharmacological effect of valerian depends on valerenic acid, which is assumed to be a ligand for GABA and adenosine receptors (20–24).In addition, many rodent studies on hops have shown results in terms of sedation and antidepressant activity as well. This has raised speculation about the activity of hops to extract on GABA receptors (25) In the United States, valerian is regulated by the Food and Drug Administration (FDA) as a dietary supplement (https://ods.od.nih.gov/factsheets/Valerian-HealthProfessional/).

The valerian-hops combination is commercially available worldwide as an OTC product (26). Valerian is considered to be relatively safe and well-tolerated, but gastrointestinal problems (e.g. nausea, abdominal cramps) are included as unpleasant side effects in the European Medicine Agency (EMA) monograph. Although hydroalcoholic extracts of valerian root in the prescribed dosage of 400-600 mg dry extract increase sleep latency and quality, the elements that contribute to its efficacy are unknown (27)

Previously published rodent studies used "anxiety-free" models and focus on valerian alone and not on the valerian-hops combination. For example, the mice receiving the methanolic and ethanolic extracts of *Valeriana Officinalis* demonstrated antidepressant and anxiolytic but no sedative effects, despite treatment for 16 days (28) A similar study reported an antidepressant effect of valerian after both an acute dosing and two weeks of chronic dosing of *Valeriana Wallichi* (29).The sedative properties of valerian, however, are not profound. Even using a dose of 1000mg/kg did not result in profound sedation (30).

According to our knowledge, no previous studies have evaluated the psychotropic actions of the valerian-hops combination versus escitalopram in a depression and in an anxiety model in association with NO_x, iNOS and SOD.

Therefore, the current research has two objectives: (1) to compare the antidepressant effect of valerian-hops with escitalopram and (2) to evaluate the sedative/anxiolytic effects of valerian-hops in relation to oxidative stress markers namely Nitric Oxide (NOx), inducible Nitric Oxide Synthase (iNOS) and Super Oxide Dismutase (SOD).

2. MATERIALS AND METHODS

2.1 Study animals and design

Balb/C mice were used for this study. All mice were six to eight weeks old and of an equal ratio of males to females. Animals were kept individually in separate cages in order to reduce anxiety as in (5)

Temperature and humidity were controlled at 25°C, 50-60% respectively.

The research followed the international ethical standards for the Care and Use of Laboratory Animals. The study protocol was approved by the Institutional Review Board (IRB) of The American University of Madaba

approval number A20001. All procedures were in an accordance with the "International Guiding Principles for Biomedical Research Involving Animals, 2012".

The study design consisted of two models:

2.2 The normal condition depression model:

Mice were divided into three groups (n=10) as follows. Group I: control (treated with a single dose of distilled water); group II: valerian-hops (treated with a single dose of 100mg/kg); and group III: Escitalopram (treated with a single dose of 10mg/kg) escitalopram was supplied from Pharma International and the dose selection was made as in (5). After a treatment period of one hour, mice were then subjected to behavioral tests.

2.3 The anxiety model:

Mice were divided into three groups (n=10) as follows. Group I: naïve (unstressed and untreated); group II: control (treated with distilled water for 3 weeks, then stressed); and group III: valerian-hops (treated for 3 weeks, then stressed). A treatment period of 3weeks was set to evaluate the antidepressant potential, as in [14]. After 3 weeks of treatment, groups II and III were restrained for six hours, then all mice were subjected to behavioral tests, blood was collected and serum was obtained, as in(5,31).

2.4 Valerian-hops combination extraction and treatments

Valerian root of (Valeriana officinalis)-hops strobile (Humulus lupulus) aqueous extract was supplied from Roha pharmaceuticals and was prepared as follows. The dry extract valerian root in a relationship of drug to extract of 4-6:1, the extraction agent was water, and dry extract of hop stabile in a relationship of drug to extract of 3-6:1, where the extraction agent was water. The extract contained a ratio of dry extract of valerian root to hop strobile of 4:1. A daily dose of 100mg/kg of valerian-hops in 0.5ml of distilled water was administered via oral gavage as in (28) for group III for 3 weeks. The dose was selected based on previous literature(32).

2.5 Acute Immobility Stress

The acute immobility stress was employed as the anxiety model according to (5,31) with slight modifications. Animals were restrained individually for 6 hours in a restraining tube, while maintaining the animals' ventilation undisturbed. In the naïve group, the mice were kept in an animal cage with soft bedding in the same experimental conditions. After performing the immobility stress, mice were subjected to behavioral tests.

2.6 Behavioral tests

2.6.1 Forced swim test:

Forced Swim Test (FST) is the most commonly used behavioral model for screening

antidepressant-like activity in rodents(33). Mice were individually forced to swim for five minutes in an open glass chamber (25×15×25 cm3), which contained freshwater to a height of 15 cm and was maintained at 26±1°C. Floating time was defined as the time in which mice stop moving completely while in the water.

2.6.2 Elevated plus maze

The anxiolytic effects were screened through the elevated plus maze as previously

described (34) with some modifications. The apparatus was elevated 25 cm above the floor.

The maze was composed of two closed arms (30*5 *10 cm) and two open arms (30*5 cm). Mice were placed at the center facing the closed arm and allowed to move freely for10 minutes. The frequency of Open Arm Exits (OAE) and the Open Arm Time (OAT) spent were recorded.

2.6.3 Open field test

The locomotion and sedation were evaluated through the standard (72*72cm) open field test as previously described (35). In brief, mice were placed in a central square and allowed to move freely for 5 minutes. The locomotion is measured by the number of lines crossed and sedation is measured by the rearing frequency. The procedure was performed in an empty room with indirect lighting, and the field was regularly cleaned with 70% ethyl alcohol.

2.7 Biochemical Tests

Performed only for the multiple-dose, anxiety-induced model in order to study stress correlation with oxidative stress.

2.7.1 Nitric oxide measurement

The accumulation of nitrate, an indicator of the production of NO, was determined in serum using a colorimetric assay with a Griess reagent (36). Serum nitrate was assayed using a Nitric Oxide Assay Kit (cat. no. ab65328; Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. The nitrate concentration was obtained according to the standard curve generated after measuring absorbance at a wavelength of 540 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples and standards were processed in duplicate.

2.7.2 SOD activity measurement

SOD activity was assayed using an SOD Assay kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; cat. no. 19160) as previously described (37) .In brief, the rate of the reduction with O2 is linearly associated with the xanthine oxidase activity, and is inhibited by SOD. The SOD activity, as an inhibition activity, is quantified by measuring the decrease in the color development at a wavelength of 440 nm.

2.7.3 iNOS measurement

The serum iNOS was measured by Abcam colorimetric kit according to the manufacturer's recommendations. The iNOS concentration was obtained according to the standard curve generated after measuring absorbance at a wavelength of 540 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples and standards were processed in duplicate.

2.8 Data and statistical analysis

All data obtained from the behavioral tests, NOx, SOD and iNOS were analyzed with a one-way ANOVA and a subsequent Tukey post hoc test, where $p < 0.05$ was considered statistically significant. Quantitative data were presented as mean values ± SEM.

3. RESULTS

3.1 The normal condition depression model

3.1.1 Forced swim test

The valerian-hops group showed lower FT compared to the control ($p<0.05$). Moreover, it showed a significant reduction in the immobility episodes and a significant increase in the floating latency time for the control group ($p<0.05$), as shown in Figure 1A, 1B and 1 C.

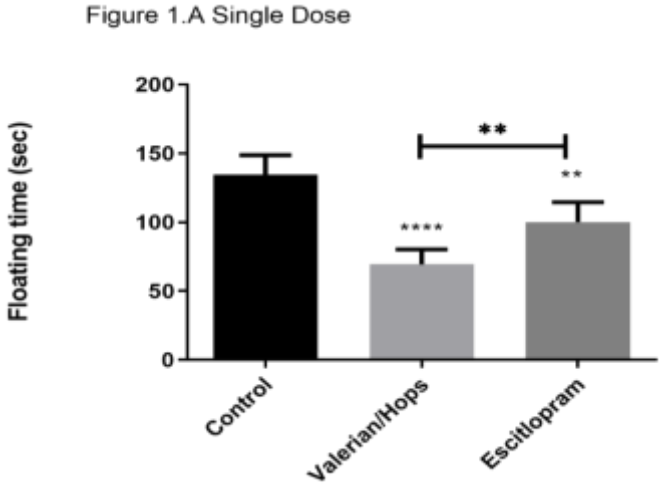
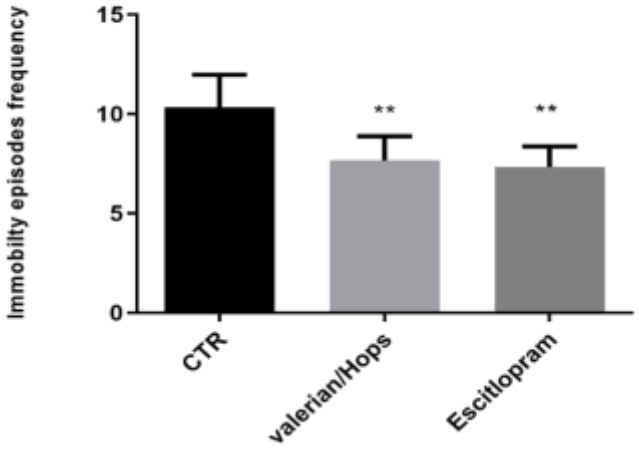


Figure 1: Forced Swim Test Single Dose Model

(A) Effect of a single dose treatment with V/H (100 mg/kg), ESC (10 mg/kg) on floating time.

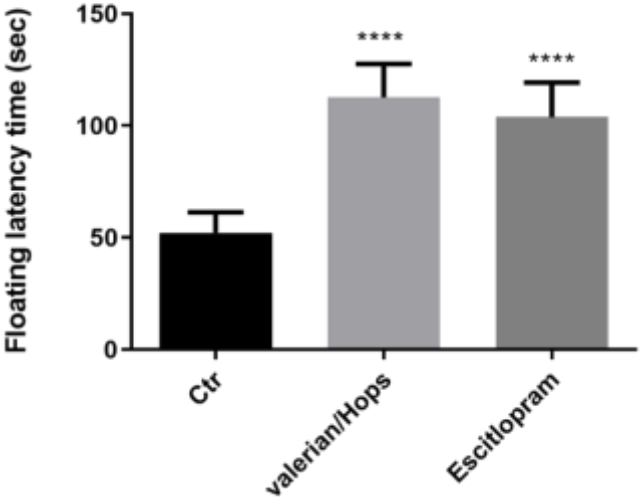
Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). F (2, 15) = 35.82 ; **p<0.001,****p<0.0001 compared to the control

Figure 1.B Single Dose



(B) Effect of a single dose treatment with V/H (100 mg/kg), ESC (10 mg/kg) on immobility episodes episodes. Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). Each bar represents the mean ± SEM (ANOVA followed by Tukey's test). F (2, 15) = 9.359; **p<0.001 compared to the control.

Figure 1.C Single Dose



(C) Effect of a single dose treatment with V/H (100 mg/kg), ESC (10 g/kg) on the floating latency time. Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). Each bar represents the mean ± SEM (ANOVA followed by Tukey's test) F (2, 14) = 29.92; **** p<0.001 compared to the control, V/H: Valerian/Hops; ESC: Escitalopram; SEM, standard error of the mean.

3.1.2 Elevated plus maze

The valerian-hops-treated group showed a significant increase in the OAE (open arm exits) count compared to the control ($p<0.05$) as seen in Figure 2.

3.1.3 Open field test

The valerian-hops-treated group showed similar ambulation frequency (AF, measured in lines crossed) to the control. Additionally, the valerian-hops-treated group showed a significantly higher rearing frequency compared to the control ($p<0.05$). Results are shown in Figure 3.

3.2 The anxiety model

3.2.1 Forced swim test

The antidepressant effect of valerian-hops combination was completely absent. The control showed a significantly higher FT compared to the naïve group ($p<0.05$). The valerian-hops group demonstrated a significantly higher FT compared to both the control and the naïve groups ($p<0.05$) and the escitalopram-treated group showed a significantly decreased FT compared to the control and naïve groups ($p<0.05$). Regarding the number of immobility episodes, the control group showed a significantly higher frequency compared to the naïve group. Both the valerian-hops-treated group and the escitalopram-treated group showed a significant reduction in the frequency compared to the control ($p<0.05$). In regards to the latency time, both the valerian-hops-treated group and the escitalopram-treated group showed a significant increase in LT compared to the control ($p<0.05$). Results are not shown.

3.2.2 Elevated plus maze

The OAE did not increase in the valerian-hops group compared to the control ($p>0.05$). However, the escitalopram-treated group demonstrated a higher OAE relative to the control group ($p<0.05$). As for open arm time (OAT) in seconds, the valerian-hops group did not show any increase compared to the control. Results are shown in Figure 2A and 2B.

Figure 2.A

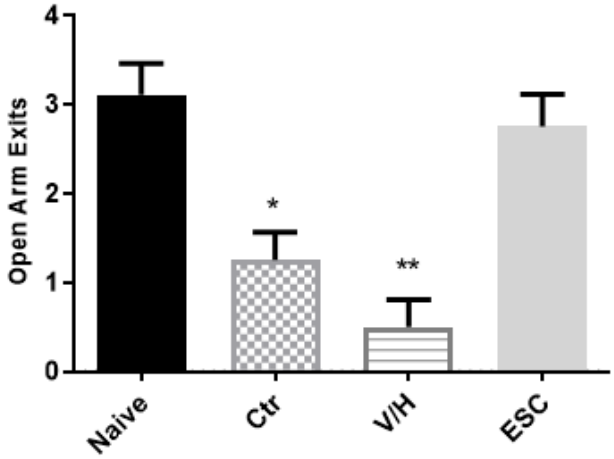
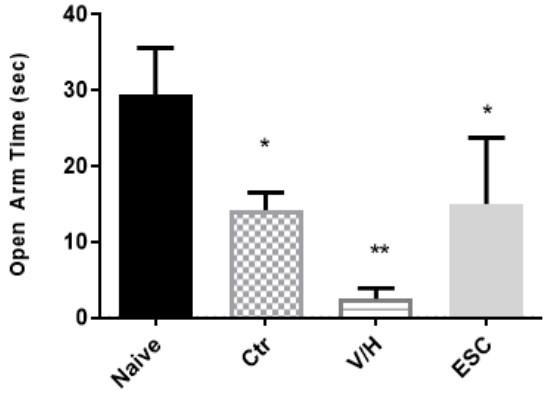


Figure 2: Elevated Plus Maze

(A) Effect of 3 weeks of treatment with V/H (100 mg/kg), ESC (10 mg/kg) on OAE following immobilization stress.

Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). F (3, 31) =14.25; *$p<0.05$, ** $p<0.0001$ versus naïve. OAE, open arm exists.

Figure 2.B



(B) Effect of 3 weeks of treatment with V/H (100 mg/kg), ESC (10 mg/kg) on OAT following immobilization stress .

ANOVA followed by Tukey's test.F (3,28)=11.10; * p<0.05, ** p<0.0001 versus naïve. OAT: open arm time. V/H: Valerian/Hops; ESC: Escitalopram; SEM, standard error of the mean.

3.2.3 Open field test

The valerian-hops-treated group did not show any increase in AF compared to the control group (p>0.05). Moreover, it significantly diminished the rearing frequency with respect to the control (p<0.05) thus indicating profound sedation. Results are shown in Figure 3A and 3 B.

Figure 3.A

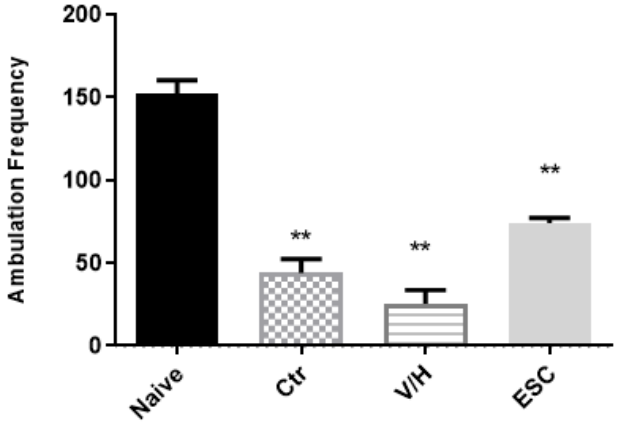
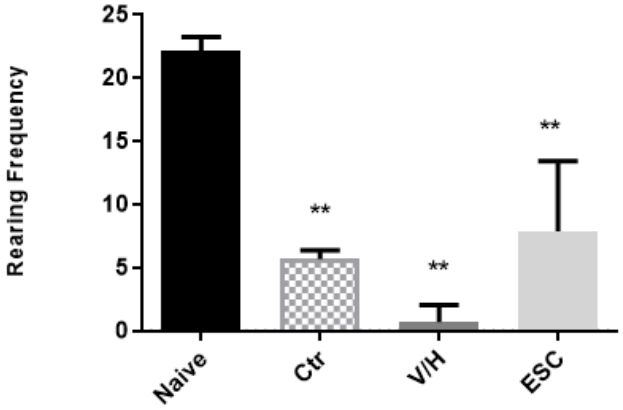


Figure 3: Open Field Test

(A) Effect of 3 weeks of treatment with V/H (100 mg/kg), ESC (10 mg/kg) following immobilization stress on the ambulation frequency.

Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). F(3,33)=57.91; **p<0.0001 versus naïve.

Figure 3.B



(B) Effect of 3 weeks of treatment with V/H (100 mg/kg), ESC (10 mg/kg) following immobilization stress on the rearing frequency.

Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). F(3, 34)=69.19;**p<0.0001 versus naïve. V/H : Valerian/Hops ; ESC: Escitalopram; SEM, standard error of the mean

3.3 Biochemical tests

3.3.1 NO_x & iNOS

The AIS increased NO_x ($p<0.05$), and this increase was significantly normalized with the valerian-hops ($p<0.001$); similarly escitalopram showed a NO_x lowering effect in respect to the stressed group ($p<0.001$), results are shown in Figure 4. The iNOS levels did not vary throughout the study (data are not shown).

Figure 4

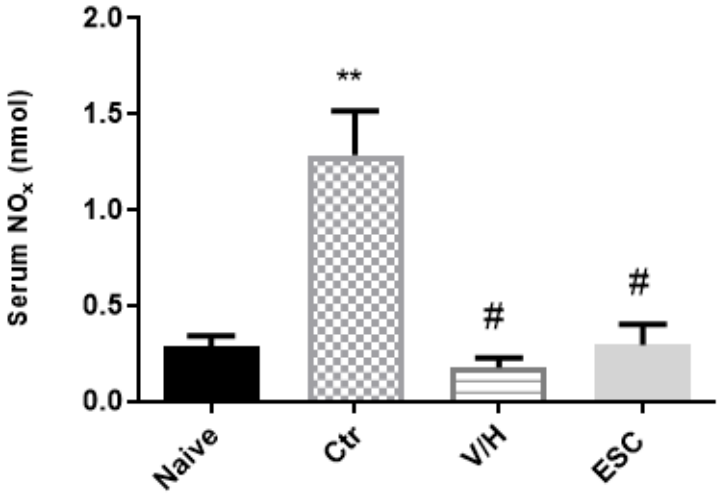


Figure 4: Nitric Oxide levels Effect of 3 weeks of treatment with V/H (100 mg/kg), ESC (10 mg/kg) following immobilization stress on serum NO_x levels.

Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). F(3, 30)=19.21;**p<0.0001 versus naïve # p<0.0001 versus control. V/H: Valerian/Hops; ESC: Escitalopram; SEM, standard error of the mean.

3.3.2. SOD activity

The AIS increased SOD activity ($p<0.05$) in the stressed group with respect to the naïve , valerian-hops treated group showed a significant decrease in respect to the stressed group ($p<0.001$); however, escitalopram did not show any change in SOD activity. Figure 5

Figure 5

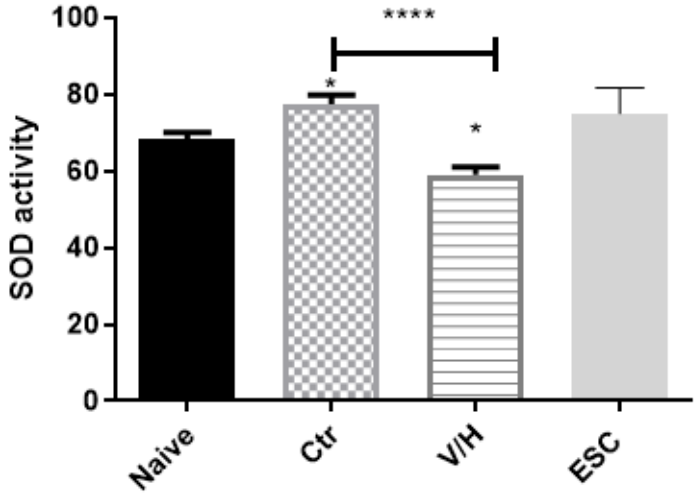


Figure 5: SOD activity

The effect of 3 weeks of treatment with V/H (100 mg/kg), ESC (10 mg/kg) following immobilization stress on SOD activity levels.

Values are expressed as the mean ± SEM (ANOVA followed by Tukey's test). F (3, 30) = 15.88;*$p<0.05$ versus naïve **** $p<0.0001$ versus control. V/H : Valerian/Hops ; ESC: Escitalopram; SEM, standard error of the mean.

4. DISCUSSION

The current study aimed at comparing the antidepressant effect of valerian-hops to escitalopram on short and long terms use using different models and to correlate the findings with NOx changes.

In the normal condition depression model, the valerian-hops demonstrated antidepressant and anxiolytic activities similar to escitalopram, but with no sedative effect. This result was expected and is consistent with previous studies that used the herb for short-term treatment(28,29). This effect is thought to be caused by agonizing the GABAA receptor, thus leading to a soothing/anxiolytic effect due to valerenic acid, the main active constituent(22,23).

The findings of the anxiety model demonstrate a profound sedative effect with no anxiolytic or antidepressant effect. These findings seem to contradict previous studies showing that valerian exerts no sedative action after five days of administration in an anxiety model(38) or after 16 days in an anxiety-free model(28). However, one explanation of this finding could be the longer duration of treatment and the synergistic sedative properties of hops(39).

The AIS resulted in a significant elevation in NOx and SOD activity in the stressed group compared to the naïve group, and valerian-hops restored NOx to normal.

As seen, the NOx elevation under AIS and the decline

by valerian-hops are not related to iNOS expression. NO can be synthesized from other enzymes; hence further investigations of the neuronal nNOS and the endothelial eNOS (9,40) are required to clarify this finding.

While the AIS enhanced SOD activity in the stressed mice, the SOD activity in the valerian-hops treated group was lower. This could be explained by the direct scavenging activity of the flavonoids and phenolic compounds in the extract(30,41) that neutralized the generated free radicals and therefore did not result in SOD activation.

The treatment of depression and anxiety is challenging due to the inadequate and inconsistent response of many classical antidepressants in addition to the associated lag and adverse effects (4,42,43).

Our findings demonstrated that the herb exerts antidepressant properties at a single dose similar to escitalopram however this effect has vanished and has been replaced by strong sedation in the anxiety model. This raises several questions behind the different mechanism underlying the two responses. Perhaps acute antidepressant property is mediated by some "fast acting" GABAergic constituents of the extract as valerenic acid and upon the chronic dosing in an anxiety model other "sedating" constituents are largely involved. The next step is to isolate and characterize the important active constituents of the valerian-hops combination.

The main strength of this study is the use of two different mice models to evaluate the antidepressant and anxiolytic/sedative role of valerian-hops. On the other hand, limitations include the use of the whole extract and the study of markers only in the anxiety model.

In conclusion, valerian-hops combination showed acute antidepressant effect similar to escitalopram when given as a single dose, whereas upon the long term use and as shown in the anxiety model valerian-hops produced only a strong sedative effect with a marked decrease in NO_x and SOD activity.

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مقارنة فاعلية مزيج عشبة النارين والجنجل بعقار الإيسيتالوبرام على نماذج قلق وإكتئاب حيوانية والعلاقة المحتملة مع الأكسدة

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ملخص

تعتبر اضطرابات الاكتئاب والقلق من أكثر مشاكل الصحة العقلية شيوعًا وترتبط بالأكسدة إتباطاً وثيقاً. على الرغم من أنه يشتهر بتأثيره المزيل للقلق، إلا أن التأثير المضاد للاكتئاب لتركيبة عشبة الجنجل لم تتم دراسته مسبقًا، كما أن العلاقة بين التأثير المهدئ لتركيبة النارين والجنجل ومؤشراتالأكسدة غير واضحة. البحث الحالي له هدفان: (1) مقارنة التأثير المضاد للاكتئاب لمزيج عشبة النارين والجنجل مع عقار الإيسيتالوبرام و(2) تقييم التأثيرات المهدئة / المزيلة للقلق لمزيج عشبة النارين والجنجل وعلاقته بمؤشرات الأكسدة وهي أكسيد النيتروجين (NOx)، إنزيم تصنيع أكسيد النيتروجين (iNOS) وإنزيم مضاد الأكسدة .Super Oxide Dismutase (SOD)تم استخدام نموذجين باستخدام الفئران :BALB / c نموذج اكتئاب للحالة الطبيعية تم فيه تقسيم الفئران إلى: مجموعة التحكم، مجموعة مزيج النارين والجنجل (100 مجم / كجم)، ومجموعات المعالجة بالإسيتالوبرام (10 مجم / كجم) قبل ساعة واحدة من الاختبار الميداني للقلق، واختبار المتاهة المرتفع للقلق، واختبار السباحة القسري للإكتئاب. النموذج الثاني هو ونموذج القلق الذي تم فيه تقسيم الفئران إلى: غير مضغوطة، والتحكم (مضغوطة)، مجموعة مزيج النارين والجنجل (100 مجم / كجم)، وإسكيتالوبرام (10 مجم / كجم). تم معالجة المجموعات لمدة ثلاثة أسابيع ؛ تم تقييده لمدة 6 ساعات وتم التضحية به، وتم الحصول على مصل للكشف عن نشاط NOx و iNOS و .SOD في نموذج الاكتئاب، أظهرت مجموعة مزيج النارين والجنجل نشاطًا مضادًا للاكتئاب مشابهًا لإسيتالوبرام. في نموذج القلق، أظهرت الفئران التي عولجت مجموعة مزيج النارين والجنجل تأثيرًا مهدئًا عميقًا في جميع نماذج السلوك، وقامت بتثبيط مستويات أكاسيد النيتروجين المرتفعة الناتجة عن القلق ونشاط انزيم ال .SOD في ظل الظروف العادية، تمارس تركيبة مزيج النارين والجنجل تأثيرًا مضادًا للاكتئاب مشابهًا لـ لإسكيتالوبرام بينما تمارس في ظروف التوتر / القلق تأثيرات مهدئة ومضادة للأكسدة.

الكلمات الدالة: القلق، الإكتئاب، إسيتالوبرام، النارين، الجنجل.

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Phytochemical Screening and *in vitro* Anti-urolithiatic Activity of Fruit-seed Extracts of *Melia azedarach*

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ABSTRACT

Melia azedarach L. has been utilized in traditional systems of medicine for the treatment of various diseases including urolithiasis. The study aimed to perform phytochemical studies and anti-urolithiatic potential of fruit-seed extracts of *Melia azedarach*. Sequential extraction was performed using chloroform, methanol and water. The extracts of the plant were then subjected to quantitative tests for phytochemical analysis. An anti-urolithiatic activity was investigated via *in vitro* nucleation and aggregation assay using spectrophotometer. The preliminary phytochemical analysis showed the presence of proteins (31.97 ± 0.56%), lipids (2.95 ± 0.03%) and carbohydrates (64.90 ± 0.56%). The chloroform extract contained the maximum quantity of polyphenols (77.65 ± 0.53 mg/g) and flavonoids (74.71 ± 0.67 mg/g). At a concentration of 5 mg/ml, the chloroform extract exhibited the maximum inhibitory activity in both aggregation and nucleation assay (55.85 ± 1.43%; 56.42 ± 4.49%) respectively. All extracts showed substantial anti-urolithiatic activity by inhibiting the crystallization of calcium oxalate. Due to presence of primary and secondary metabolites, the plant could serve as a source of useful drugs.

Keywords: *Melia azedarach*, phytochemical analysis, anti-urolithiatic, aggregation assay, nucleation assay.

1. INTRODUCTION

Urolithiasis or renal stone disease remains a serious issue in the adult population, with serious medical consequences throughout life (1). The prevalence of urolithiasis is quite high around the globe and more than 80% of urinary calculi are calcium oxalate stones alone or calcium oxalate mixed with calcium phosphate (2, 3). Despite modest progress in the pathophysiology and treatment of urolithiasis, there is still no effective drug being utilized in clinical therapy (4). Extracorporeal shock-wave lithotripsy (ESWL) and endoscopic stone removal are well-recognized procedures but it is expensive and recurrence is quite common with these procedures (5). However, an effective drug for the treatment of this disorder or its recurrence would be of great importance. Medicinal plants have played a substantial role in various ancient traditional systems of medicine. Several pharmacological investigations on the medicinal plants used in traditional anti-urolithiatic therapy have revealed their therapeutic potential in the *in vitro* models. Various medicinal plants exhibiting anti-urolithiatic activity have been well documented in previous studies (6-9).

Melia azedarach, a deciduous tree is derived from Greek words; *Melia* means "flowering ash or manna ash" and *azedarach* means "poisonous tree" (10). It is commonly used in Chinese, Iranian and Indian traditional medicines (11). All parts of the plant i.e. root, bark, leaves, seeds, flowers, and fruits have shown pesticidal as well as pharmacological activities (10). It is well-known for its antioxidant, cardioprotective, anti-inflammatory, anti-

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microbial, anti-ulcer and anti-cancer properties which are proven by clinical and experimental studies (12-15). The literature review of *Melia azedarach* revealed that this plant contains many phytochemicals that possess various pharmacological activities (16). Various studies have been focused on leaves, bark, flower, and seeds but fruit-seed is not so much focused. However, more exploratory research is required to evaluate safety, efficacy and therapeutic potential of this plant. Therefore, the aim of this study is to assess phytochemical studies and anti-urolithiatic activity of fruit-seed extracts of *Melia azedarach*.

2. MATERIALS AND METHODS

2.1. Chemical and reagents

Chloroform, methanol, acetone, potassium acetate, aluminium nitrate crystals, Bovine Serum albumin (BSA), copper sulphate, potassium tartrate, sodium bicarbonate (Merck, Germany), Cystone (Himalaya, India), sodium oxalate, calcium chloride (Duksan Reagents, Korea), Tris-HCl (Sigma, Aldrich, Germany), hydrochloric acid, sulphuric acid (BDH, England).

2.2. Plant material and extraction

In December 2019, the fruit-seeds were collected from Akbari Mandi, Lahore, Pakistan. This plant was then authenticated by taxonomist Prof. Dr. Zaheer-ur-Din Khan, Department of Botany, Government College University Lahore (GCU), Pakistan. A voucher specimen (GC Herb. Bot. # 3645) was deposited in the herbarium of GCU. The fresh fruit-seeds were separated, cleaned and air-dried under shade for 2 weeks at room temperature. The dried fruit-seeds were compressed into a fine powder and stored in an air-tight bag. It was then subjected to proximate analysis.

The powdered material (50 g) was extracted sequentially using solvents of different polarities i.e. chloroform, methanol and water. All the extracts were dried with the help of a rotary evaporator, keeping the temperature less than the boiling point of the respective solvent. The extracts were collected in tarred, cleaned and labeled storage vials, and allowed to dry in the oven at 40 ℃ until the solvent disappeared.

2.3. Phytochemical analysis

2.3.1. Estimation of Primary Metabolites

The different extracts of *Melia azedarach* fruit seed were evaluated for the quantification of primary metabolites such as carbohydrates, proteins, and total lipids.

2.3.1.1. Estimation of total lipids

Continuous hot extraction of the plant was performed to determine the lipid content (17). Petroleum ether was used as a solvent. Before extraction, the thimble was macerated in an organic solvent for about 12 hours and then extraction was performed in Soxhlet apparatus at 40℃ - 60℃ for 24 hours. The extract was then filtered and dried in the rotary evaporator at 50℃ up to 10 ml. It was then stored in an oven so that extract was dried. After drying, the weight of the extract was determined to calculate the total lipid contents.

2.3.1.2. Estimation of total proteins

Total protein contents of *Melia azedarach* fruit seed were evaluated according to the protocol prescribed by Lowry et al. (1951) (18). About 1 g of fruit-seed powder was weighed accurately. The sample was then mixed with 10 ml of distilled water along with 2-3 drops of Triton-X and macerated for about thirty minutes. It was then centrifuged at 2700 rpm for at least 10 minutes. A 100 µl of the supernatant was collected in a falcon tube and made-up volume up-to 1 ml with distilled water. About 3 ml of reagent C that was prepared by mixing 50 ml of reagent A (composed of mixing 2% sodium carbonate in 0.1N sodium hydroxide) and 1ml of reagent B (composed of mixing 0.5% copper sulphate in 1% potassium sodium tartrate)) was added in the falcon tubes. After adding reagent C, 200 µl of Folin-Ciocalteu reagent was added in the respective falcon tubes and incubated for 30 minutes at room temperature. To plot the standard curve, Bovine Serum Albumin (BSA) of different concentrations was used as a standard. Blank was prepared using the same method that except standard and sample. The absorbance

of plant and standard was measured at 600 nm. Total protein was performed in triplicated and evaluated from standard curve by using linear regression equation.

2.3.1.3. Estimation of total carbohydrates

Total carbohydrates were then evaluated by the following formula as mentioned in Al-Hooti (2008) (19).

Total carbohydrates (%) = 100 - (total protein + total ash + total fat + moisture content)

2.3.2. Estimation of Secondary Metabolites

2.3.2.1. Estimation of polyphenols

Total polyphenol contents of fruit seed of Melia azedarach were evaluated according to protocol prescribed by Singleton and Slinkard (1997) (20). A methanolic solution of Gallic acid (mg/ml) of different concentrations was used as a standard to plot standard calibration curve. Accordingly, 200 µl of standard/test solution was taken into falcon tubes. A 200 µl of Folin-Ciocalteu reagent was added in the respective falcon tubes and mixed the solution homogenously. After five minutes, 1ml of 7.5% of sodium carbonate was added into the falcon tubes and the final volume was made up to 3 ml with methanol. Blank was treated like test solution/standard with the exception of standard or test solution which is replaced by methanol. The reaction mixture was incubated at room temperature for about 2 hours and absorbance was then measured at 760 nm. Standard curve of gallic acid was plotted and polyphenol content was determined from the calibration curve of the standard. The phenolic contents were expressed as mg/g of gallic acid equivalent by the linear regression equation.

2.3.2.2. Estimation of total flavonoids

Pavun *et al* (2018) method was used for the determination of the total flavonoids with little modification (21). A methanolic solution of quercetin (mg/ml) of different concentration was used as a standard to plot the standard calibration curve. A reaction mixture contains 200 µl of standard/test solution, 100 µl 10% aluminium nitrite and 100 µl of 1M potassium acetate and final volume was made up to 5 ml with distilled water. Falcon tubes were then incubated for 30 minutes at room temperature and absorbance was measured at 415 nm. The standard curve of quercetin was plotted and flavonoid content was determined from the calibration curve of standard.

2.3.2.3. Estimation of polysaccharides

To determine the total polysaccharide content, the protocol recommended by Hussain et al. , was used with little modification (22). A 200 mg of each extract was mixed with 7ml of hot ethanol (80%) in falcon tubes to eliminate any soluble sugars. The reaction mixture was vortexed for 5 minutes and then centrifuged at 2700 rpm for 10 minutes. The supernatant layer was discarded. The residue was washed with anthrone reagent (0.2% in concentrated sulphuric acid) until the color disappeared and placed into the water bath until the residue was dried. After that, 5 ml of HCl (25%) and 5 ml of distilled water were added into respective falcon tubes. The falcon tubes were then incubated at 0°C for 25 minutes and centrifuged at 2700 rpm for 10 minutes. This process was repeated three times and the supernatant was collected in a 100ml volumetric flask final volume was made with distilled water.

Then, 100 µl of supernatant was transferred into the test tubes and the final volume was made up to 1 ml with water and 4 ml of anthrone reagent was then added. The reaction mixture was then heated in a boiling water bath for eight minutes and allowed it to cool. The absorbance of the reaction mixture was measured at 630 nm. The glucose was used as standard and total polysacharrides were evaluated by multiplying the glucose content by 0.9.

2.3.2.4. Estimation of glycosaponins

Total glycosaponins were estimated according to the protocol as prescribed by Hussain et al. 2008 (22). Accordingly, 1 g of different extracts was refluxed in 50 ml methanol for 30 minutes. The process was repeated until all the glycosaponins were extracted. The extracts were then concentrated up to 10 ml through a rotary evaporator. After that, the concentrated extract was added into the beaker containing 50ml of acetone. The precipitates were obtained and dried in an oven at 60°C.

The weight of precipitates was measured to calculate the total glycosaponins by using the following formula;

$$\text{Total glycosaponins} = \frac{\text{Weight of precipitates}}{\text{Weight of sample}} \times 100$$

2.4. Anti-urolithiatic activity

2.4.1. Aggregation assay

The anti-urolithiatic activity was determined using aggregation assay (23). Calcium oxalate was prepared by mixing equimolar calcium chloride solution (50 mM) and sodium oxalate solution (50 mM) and allowed it to stand overnight for the formation of calcium oxalate crystals. The crystals were then washed with ethanol twice to remove any impurities. It was then filtered and placed in the oven at 60 ℃ for 1 hour. Calcium oxalate solution was prepared in Tris-HCl buffer at a concentration of 8 mg/ml at pH 6.5. The reaction mixture contains 1 ml of extracts (5 mg/ml) and 2 ml of calcium oxalate solution. The test was performed at 37 ℃. The absorbance was measured at 620 nm for 30, 60, 180 and 360 minutes. Cystone was used as a standard. Control is treated like the test solution except standard/extract solution which was replaced by the solvent used. The turbidity was calculated by using the following formula;

$$\text{Turbidity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

2.5. Nucleation assay

Nucleation assay was assessed according to the published protocol with little modification (23). The solution of calcium chloride (4 mM) and sodium oxalate (7.5 mM) was prepared in tris HCL buffer (50 mM Tris-HCl and 150 mM NaCl) at pH 6.5. A volume of 1 ml extract (5 mg/ml) was mixed with 1ml of calcium chloride solution and incubated at 37 ℃ for 5 minutes. A volume of 1 ml sodium oxalate solution was then added to induce the reaction of crystallization. The test solution was maintained at 37℃. The absorbance was measured at 620 nm for 30, 60, 180 and 360 minutes. Cystone was used as a standard. A control is treated like test solutions with the exception of standard/extract solution which is replaced by solvent used. The turbidity was calculated by using the following formula;

$$\text{Turbidity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

2.6. Statistical analysis

All the tests were performed in triplicate and the means were calculated. All the values were expressed as means $\pm$ standard deviation (SD). A t-test was utilized to test for comparing two groups. A p-value <0.05 was considered to indicate statistical significance.

3. RESULTS AND DISCUSSION

3.1. Phytochemical screening

The standard curves of Bovine Serum Albumin (BSA), quercetin, gallic acid and glucose were plotted for the estimation of total proteins, flavonoids, polyphenols and polysaccharides (Figure I-IV).

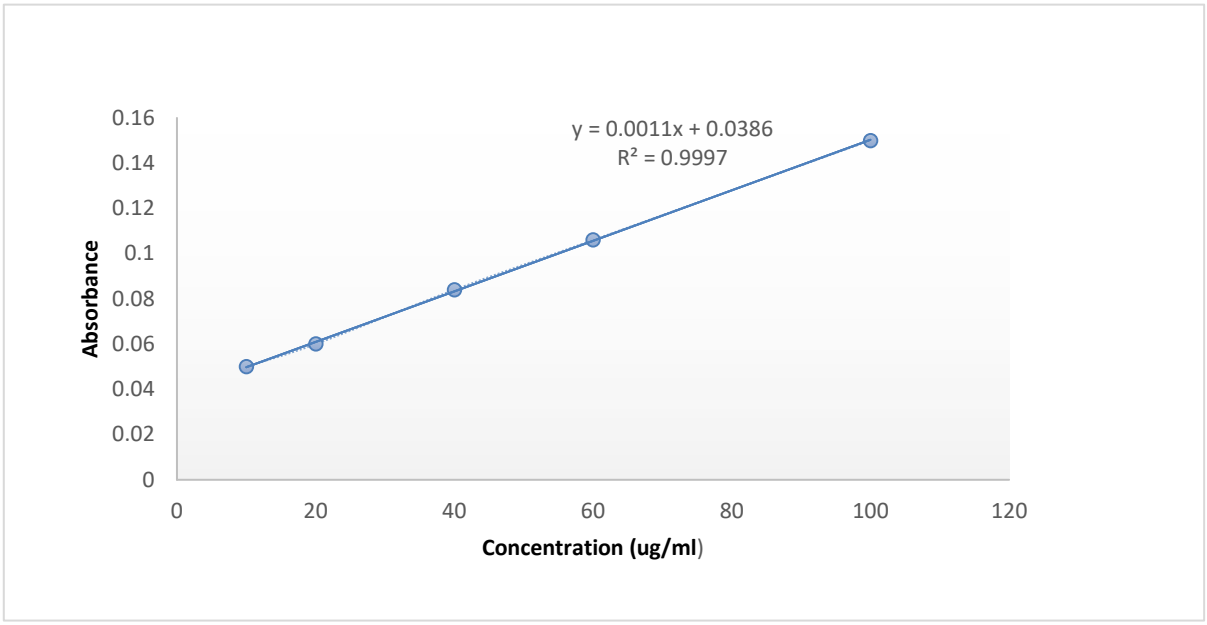

Figure I: Standard curve of Bovine Serum Albumin (BSA) for the estimation of proteins.

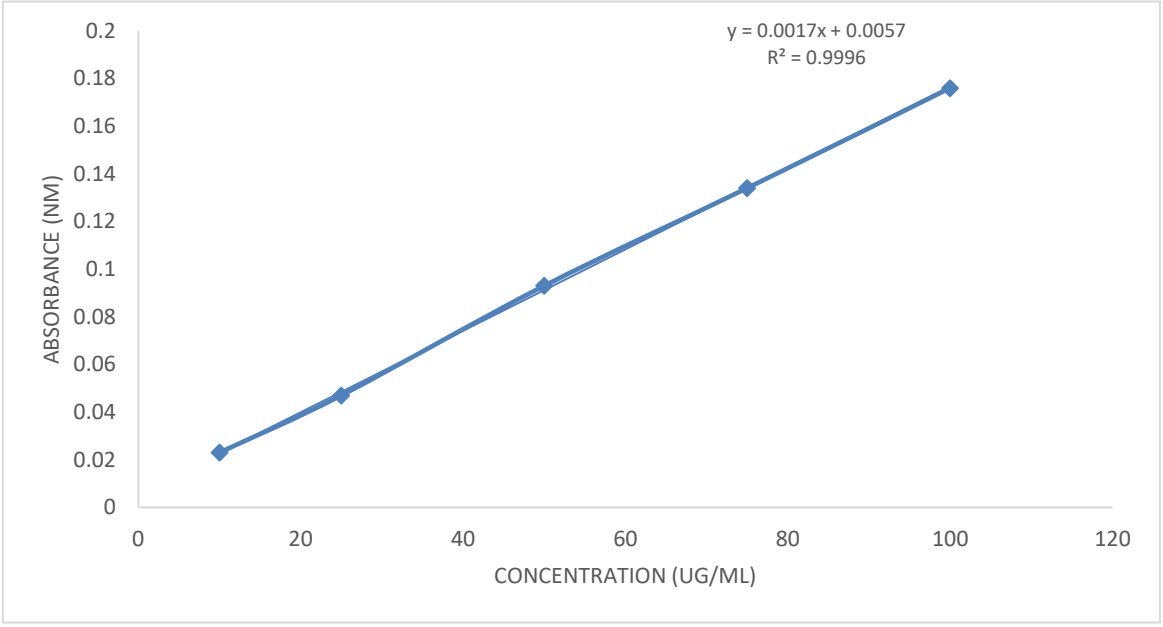

Figure II: Standard curve of Quercetin for the estimation of flavonoids.

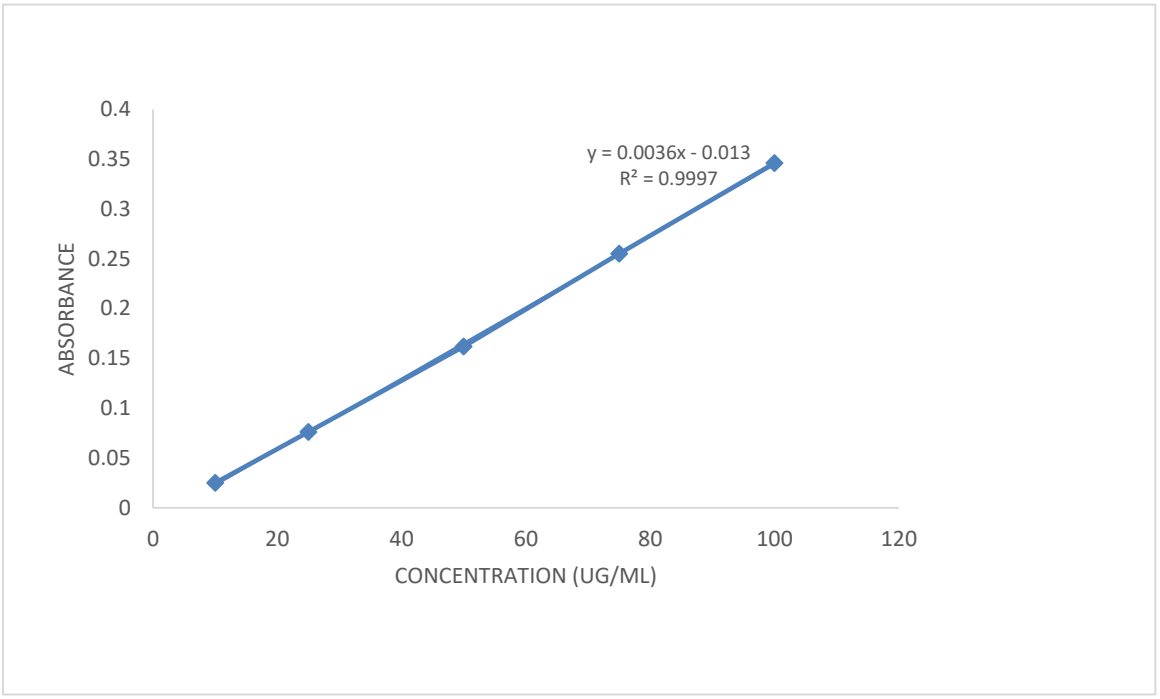

Figure III: Standard curve of Gallic acid for the estimation of polyphenols.

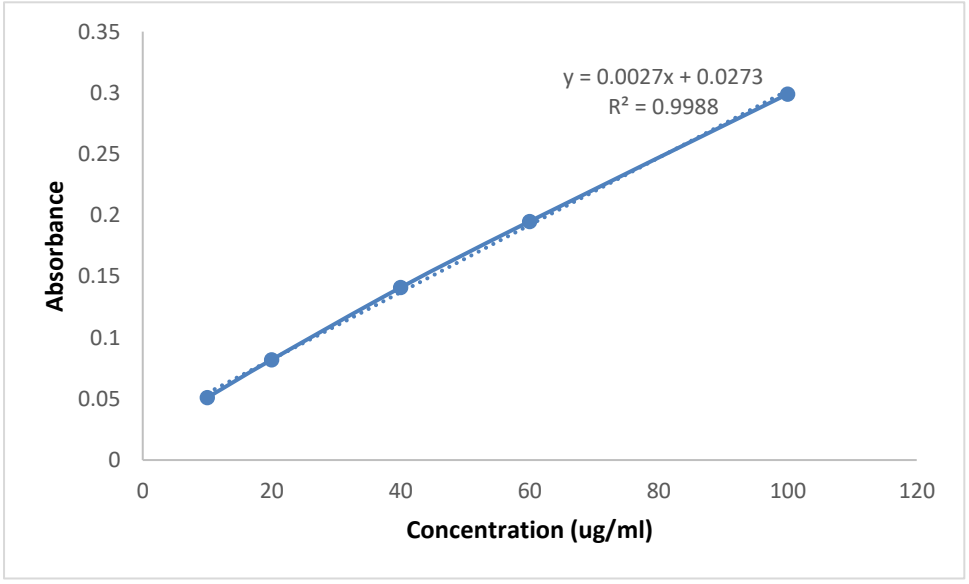

Figure IV: Standard curve of glucose for the estimation of polysaccharides.

Melia azedarach reported the presence of total proteins (31.97 ± 0.56%), total lipids (2.95 ± 0.03%) and total carbohydrates (64.90 ± 0.56%) as shown in Table I. The findings of secondary metabolites (total polyphenols, total

flavonoids, total polysaccharides and total glycosaponins) were summarized in the Table II. The chloroform extract showed the maximum quantity of polyphenols (77.65 ± 0.53 mg/g) and flavonoids (74.71 ± 0.67 mg/g). However, a large amount of glycosaponins (42.71 ± 0.53 mg/g) and polysaccharides (125.27 ± 0.41 mg/g) were observed in aqueous extract. Phytochemical screening is essential for the identification of lead components that possess pharmacological activities (24). These include primary (proteins, carbohydrates, and lipids) and secondary metabolites (Polyphenols, tannins, flavonoids, alkaloids and saponins) (25). Our present study reported the presence of phytochemical constituents in *Melia azedarach*. Another study reported the presence of proteins, carbohydrates, lipids, glycosides, flavonoids, and polyphenolic compounds in plant (26). The maximum quantity of polyphenols and flavonoids was present in chloroform extract. However, Ahmed and his colleagues documented that the alcoholic extract had large quantity of polyphenolic compounds, followed by aqueous and petroleum ether extracts (27). A variety of phytochemical constituents was found in plants that exhibited pharmacological and therapeutic activities.

Table I. Total primary metabolites in fruit-seed powdered material of *Melia azedarach*.

Primary Metabolites	Percentage content ± SD
Total protein	31.97 ± 0.56
Total lipids	2.95 ± 0.03
Total carbohydrates	64.90 ± 0.56

Table II. Total content (mg/g) of secondary metabolites of fruit-seed extracts of *Melia azedarach*.

Extracts	Total Polyphenols (mg/g)	Total Flavonoids (mg/g)	Total Glycosaponins (mg/g)	Total Polysaccharides (mg/g)
Chloroform	77.65 ± 0.53	74.71 ± 0.67	3.86 ± 0.04	6.38 ± 0.42
Methanol	43.02 ± 0.72	23.06 ± 0.69	11.21 ± 0.03	86.47 ± 0.49
Aqueous	23.32 ± 0.53	10.31 ± 0.33	42.71 ± 0.53	125.27 ± 0.41

3.2. Anti-urolithiatic activity

The effect of inhibition of aggregation and nucleation activities for various plant extracts were illustrated in Figure V and VI. All the extracts showed increasing trends with an increase in incubation time. In aggregation assay, the chloroform extract (55.85 ± 1.43%) possessed more potency in the dissolution of calcium oxalate crystals, followed by methanolic extract (48.58 ± 3.48%) and aqueous (45.74 ± 5.37%) after the incubation of 360 minutes (Figure V). Similarly, in nucleation assay, chloroform extract exhibited highest inhibitory activity (56.42 ± 2.39%), followed by methanolic (54.10 ± 4.49%) and aqueous (39.70 ± 0.76%) extract (Figure VI).

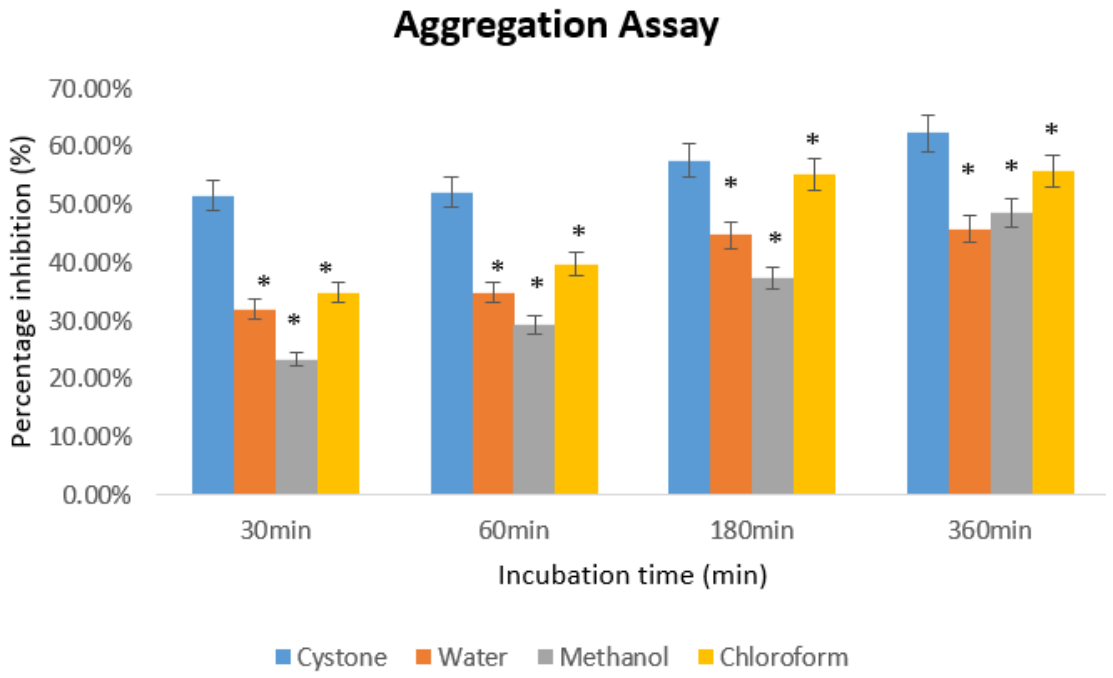

Figure V: Aggregation assay of different extracts of fruit-seed of *Melia azedarach* and positive control (cystone). Bars with asterisk (*) are significantly different from standard (Cystone), p<0.05.

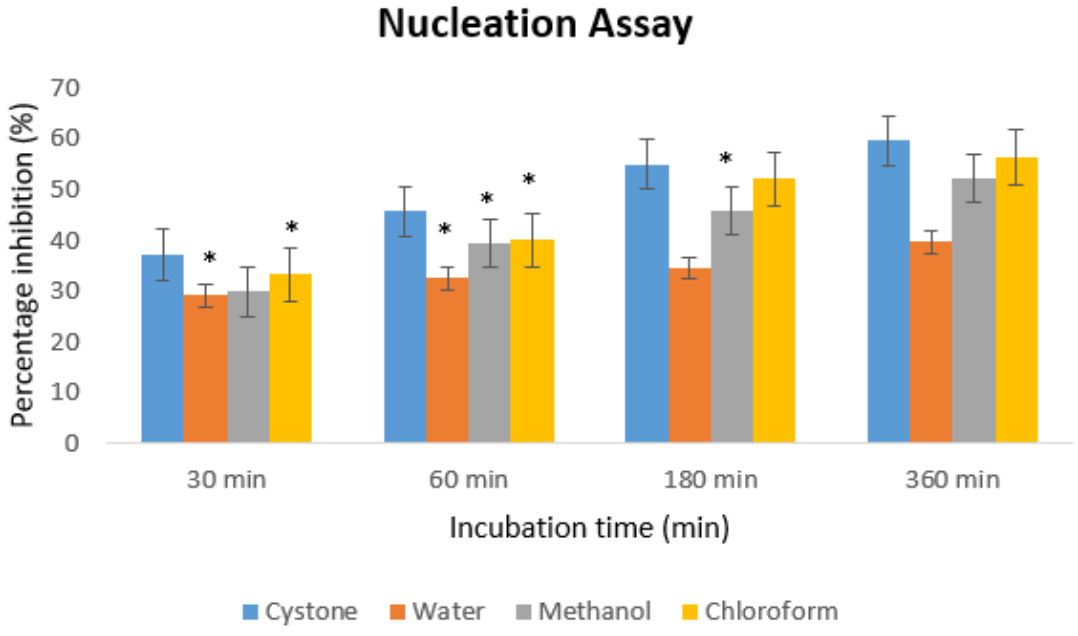

Figure VI: Nucleation assay of different extracts of fruit-seed of *Melia azedarach* and positive control (cystone). Bars with asterisk (*) are significantly different from standard (Cystone), p<0.05.

In aggregation assay, the difference in activity between methanolic extracts and control was statistically significant ($p<0.05$). However, in nucleation assay, the activity between extracts and control was not statistically significant after the incubation of 360 minutes ($p>0.05$). The order of inhibitory activity of various plant extract were cystone > chloroform > methanol > aqueous.

Due to presence of various phytochemicals, *Melia azedarach* showed the anti-urolithiatic activity. However, a study reported that alcoholic and aqueous extracts of plant leaves exhibited highest inhibitory activity against ethylene-glycol-induced calcium oxalate urolithiasis in male albino Wistar rats (1). It increased the urine volume and urinary magnesium levels and minimized the level of urinary phosphate, oxalate and calcium. Similarly, another study reported the anti-urolithiatic activity in alcoholic extracts of plant leaves (3). The results may differ due to geographical origin of the plant. However, the mechanism of action is still not clear. It could be due to the presence of antioxidant, antilithiatic, diuretic, and anti-inflammatory constituents in plant. There is no data available on fruit-seed extract of *Melia azedarach*.

4. CONCLUSION

M. azedarach was found to be rich in primary as well as secondary metabolites which represents that there will be a high content of potential bioactive compounds to treat number of diseases. The chloroform extract showed the high concentration of polyphenols and flavonoids. The outcomes of the present study reveals that *M. azedarach* has a potential to prevent the aggregation and nucleation of calcium oxalate crystals. However, further investigation is needed to assess the mechanism of action in animal models of lithiasis. The positive outcomes may be due to the presence of phytochemicals, thus, further characterization, isolation of bioactive components from plant extracts are needed. Extensive research is required to characterize and isolate bioactive constituents and evaluate other mechanisms of action.

Conflicts of interests

None.

Acknowledgments

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فحص كيميائي نباتي ونشاط مضاد لمجرى البول في المختبر لمستخلصات بذور الفاكهة من ميليا أزدارش

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ملخص

تم استخدام ميليا أزداراش في أنظمة الطب التقليدية لعلاج العديد من الأمراض بما في ذلك تحص بولي. هدفت الدراسة إلى إجراء دراسات كيميائية نباتية وإمكانات مقاومة المسالك البولية لمستخلصات بذور الفاكهة من ميليا أزداراش. تم إجراء الاستخراج المتسلسل باستخدام الكلوروفورم والميثانول والماء. ثم خضعت المستخلصات النباتية لاختبارات كمية لتحليل الكيمياء النباتية. تم التحقيق في نشاط مضاد لمجرى البول عن طريق التنوي في المختبر ومقايسة التجميع باستخدام مقياس الطيف الضوئي. أظهر التحليل الكيميائي النباتي الأولي وجود بروتينات (31.97 ± 0.56٪)، دهون (2.95 ± 0.03٪) وكربوهيدرات (64.90 ± 0.56٪). يحتوي مستخلص الكلوروفورم على أقصى كمية من البوليفينول (77.65 ± 0.53 مجم / جم) والفلافونويد (74.71 ± 0.67 مجم / جم). بتركيز 5 ملغ / مل، أظهر مستخلص الكلوروفورم أقصى نشاط مثبط في كل من التجميع ومقايسة النواة (55.85 ± 1.43٪ ؛ 56.42 ± 4.49٪) على التوالي. أظهرت جميع المستخلصات نشاطا كبيرا في مقاومة المسالك البولية عن طريق تثبيط تبلور أكسالات الكالسيوم. بسبب وجود المستقلبات الأولية والثانوية، يمكن أن يكون النبات بمثابة مصدر للأدوية المفيدة.

الكلمات الدالة: ميليا أزداراش، التحليل الكيميائي النباتي، مضاد للجراثيم، فحص التجميع، فحص النواة.

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Optimization and Validation of HPLC-UV Method for the Determination of Vardenafil, Sildenafil, and Tadalafil in Honey-Mixed Herbal Sachets Using a Design of Experiment

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ABSTRACT

A method was developed for the simultaneous determination and analysis of sildenafil, vardenafil, and tadalafil in honey-mixed herbal sachets using high-performance liquid chromatography with a UV detector (HPLC–UV). This method eliminates the employment of complex procedures and abolishes time-consuming and labour-intensive pre-treatment processes. In ten minutes, the separation process (at 25oC) of sildenafil, vardenafil, and tadalafil using a C18 150 mm × 4.6 mm x 5 µm column (Shim-pack GIST) was successful with high selectivity and sensitivity. The mobile phase was a 60:40 (v/v) mixture of 0.1 percent formic acid in water and 0.1 percent formic acid in acetonitrile. Using the mobile phase as an extraction mixture, it gave recoveries in the range of 93.0-103.3% at spike levels of 50–150 mg/kg with relative standard deviations (RSDs) lower than 10%. The intra-day and inter-day precision results were in the range of 0.4–0.8% and 1.0–1.7%. Furthermore, the retention times for sildenafil acid, vardenafil acid, and tadalafil were 1.93, 2.47, and 9.62 minutes, respectively, and the limits of detection (LOD) were 1.70, 2.16, and 1.03 mg/L, while the limits of quantification (LOQ) were 5.65, 7.21, and 3.42 mg/L.
Keywords: HPLC, experiment design, sildenafil, vardenafil, tadalafil, a PDE_5 inhibitor.

INTRODUCTION

Erectile dysfunction (ED) is the inability to sustainably achieve or maintain an erection sufficient for satisfactory sexual performance [1]. This prevalent condition is common in men over the age of 40 and can have a significant impact on quality of life and self-esteem.

In the past, due to limited understanding of the physiological mechanism of erection, treatment of ED was limited to vacuum contractors, prosthetic implants, intracavernosal injections, and intraurethral suppositories [2]. Since its emergence, a class of drugs known as type 5 phosphodiesterase (PDE_5) inhibitors has revolutionized the treatment of ED. PDE_5 inhibitors have become the first-line therapy for ED recommended by the American Urological Association (AUA) and the European Association of Urology (EAU) [2, 3].

In the mid-1980s, the relationship between nitric oxide (NO) and the PDE family increased drug innovation. Many physiological effects of NO have had dramatic effects on many illnesses. PDE enzymes are ubiquitous in the body, and 11 recognized isozymes are expressed at different levels in different tissues. The PDE5 enzyme is widespread

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but more common in penile tissue. Before discovering the association between NO and PDE, non-selective PDE inhibitors (such as theophylline) were used, but selective PDE inhibitors have not yet been developed. Since then, several selective PDE inhibitors have been approved to treat conditions ranging from ED to pulmonary hypertension [4].

Four oral PDE5 inhibitors commercially available in the United States are sildenafil® (Viagra, Pfizer), vardenafil® (Levitra and Staxin, Bayer / GlaxoSmithKline), tadalafil® (Cialis, Eli Lily), and recently approved. It is a drug, avanafil® (Stendra), Vivis). This class of extensions gives you more flexibility when prescribing based on individual responses. During sexual arousal, NO is released from the corpus cavernosum nerve endings and endothelial cells. NO activates guanylate cyclase to convert guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), triggering cGMP-dependent events. Accumulation of cGMP leads to relaxation of smooth muscle in the corpus cavernosum and increased blood flow to the penis [5, 6].

The most frequently reported adverse events with oral PDE_5 inhibitors are headache, flushing, dyspepsia, dizziness, and rhinitis [5, 7-11]. All PDE_5 inhibitors currently available are highly selective for the type 5 gene family. However, sildenafil and vardenafil are less selective for PDE_6 expressed in the retina [12, 13]. Patients have reported vision-related side effects associated with PDE_6 inhibition, including dose-dependent disorders of color discrimination (blue/green) or cyanopsia (objects appear blue) [14-16]. In contrast, tadalafil is selective for PDE6 and PDE_{11}, which is concentrated in the prostate, testis, and skeletal muscle [12, 13]. Inhibition of type 11 PDE isozymes is associated with pain and myalgia [17]. Analytical techniques for analyzing PDE_5 inhibitors and their analogs have been developed for public health safety and regulation [18-20]. In this case, various spectroscopic and chromatographic methods are used to inspect illegally grown foods containing undeclared synthetic drugs in a complex matrix. The most common techniques for measuring PDE5 inhibitors and their analogs are UV-Vis detectors (UVs) [21-23], spectrophotometers [24], and gas chromatography combined with mass spectrometry (High-Performance Liquid Chromatography (HPLC) with GC-MS) [25]. Recently, liquid chromatography (LC-MS) combined with mass spectrometry and liquid chromatography (LC-MS / MS) combined with tandem mass spectrometry have been adopted [19, 26-28]. High-Performance Liquid Chromatography (HPLC-PDA) with a diode array detector can identify the analyte by comparing the UV spectra of the reference compound and the target compound. In this regard, MS has a great deal of interest in qualitative and quantitative analysis[29]. Especially in quantitative analysis, multiple reaction monitoring (MRM) of triple quadrupole mass spectrometry (QQ MS) shows high sensitivity and remarkable property selectivity[30].

This study aims to develop an easy and quick way to determine the presence or absence of sildenafil, vardenafil, and tadalafil in herbal sachets mixed with honey. It uses a simplified solvent extraction method followed by HPLC with a UV-Vis detector. The proposed method is internally validated for linearity, daytime and daytime accuracy, LOD, and LOQ.

EXPERIMENTAL SECTION

Materials

Certified standard solutions of sildenafil (98.0 %), vardenafil (98.0 %), and tadalafil (98.0 %) were procured from AmBeed, Illinois, United States. Daily working standard solutions were prepared by diluting the stock solutions in the mobile phase. Ammonium formate was purchased from Agilent Technologies (USA). Formic acid (99.8 %) was obtained from Fluka (Buchs, Switzerland). Water was purified using reverse osmosis technology and an Electrodeionization (EDI) system (Maxima Ultra-Pure Water, England). A non-sterile PTFE Syringe Filter with a disposable membrane filter (0.45 μm) was purchased from

Whatman GmbH (Dassel, Germany).

Instrumentation

The HPLC analysis was performed using a Shimadzu LC-2030C system (Kyoto, Japan) consisting of a degasser, four-solvent low-pressure gradient pump, autosampler, column oven, a UV-Visible detector, and an autosampler equipped with a 200-µL sample loop and controlled by the Lab Solution system (version 5.90, Shimadzu, Japan). The chromatographic separation was performed with Shim-pack GIST, C18, 5 µm, 4.6 x 150 mm chromatographic column purchased from Shimadzu Corporation (Kyoto, Japan). In the mobile phase, the sample extracts were analyzed using 0.1% formic acid in water and 0.1% formic acid in an acetonitrile 60:40 (v/v) mixture. The column was kept in a column oven at 25° C at a flow rate of 0.8 mL/min to achieve the optimum resolution between PDE_5 inhibitors. The injection volume was maintained at ten µL for both sample and standard solutions. The wavelength at 220 nm was applied to detect all PDE_5 inhibitors.

Procedure

Sample preparation

Honey samples were prepared using a solid-liquid extraction procedure described below [31].

Step I: A thoroughly homogenized honey sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 mL).

Sample recovery was made with 1.0 g of the non-contaminated honey samples with three different fortification levels; 0.5 mL of PDE_5 inhibitors mixed standards were spiked at 5.0, 10.0, and 20.0 µg/kg of honey. The spiked samples were left overnight (14 h) in the dark at room temperature (humidity between 30% and 60%) to allow the solvent to evaporate and for PDE_5 inhibitors absorption into the matrix. Then they were extracted via the following steps (II to III).

Step II: 10.0 mL of 50:50 (v/v) acetonitrile/water mixture was added to the fortified samples, and the centrifuge tube was manually shaken vigorously for 1 min to ensure that the solvent had mixed thoroughly with the entire sample. Therefore, the extraction of the analyte was complete.

Step III: The extract was centrifuged for 5 min at 4000 rpm. 1.0 mL of the upper organic layer was filtered through a 0.45 µm nylon syringe filter before HPLC analysis.

Preparation of standard stock solution and calibration standard.

The standard stock solution of PDE_5 inhibitor (1000 mg/L) was prepared with the mobile phase. A series of standard solutions (12.50, 25.00, 50.00, 100.0, and 200.0 mg/L) was prepared by diluting adequate volumes of the PDE_5 inhibitors stock solution with the mobile phase.

Optimization procedure

The pH of the mobile phase and mobile phase ratio plays a significant role in chromatographic separations. The DOE and statistical data analysis were performed using Minitab® 20.0 software system. For the robustness study, a CCD with twenty-six experimental points (Table 1) was performed randomly at all points. Resolution (R_S), in addition to the maximum peak, was chosen as the response of the food PDE_5 inhibitors.

Table 1: The effects of the acetonitrile percentage, flow rate, and format amount on the chromatographic peak areas for PDE_5 inhibitors, on retention of tadalafil, and the resolution between vardenafil and sildenafil analysed by Zorbax Eclipse XDB-C18 150 mm × 4.6 mm column at 285 nm at room temperature.

Run Order	0.1% Formic Aid (v/v) Acetonitrile	Flowrate	Formate amount	Area VAR	Area SIL	Area TAD	TAD retention time (min)	Resolution between VAR & SIL
1	45	1.2	0	5596588	3716224	4203470	3.266	0.84
2	35	1	10 mM	7347353	4717276	5933050	7.619	2.30
3	25	0.8	0	11088708	11192518	7678229	11.657	5.00
4	45	1.2	10 mM	7163758	7475723	7475723	3.803	0.81
5	25	1.2	10 mM	7732072	5317255	10611312	46.991	2.90
6	45	0.8	10 mM	7117393	5175559	5834347	3.925	0.86
7	45	0.8	0	7078510	5173909	5810842	3.926	0.87
8	35	1	0	8794939	6003490	8779507	7.598	2.20
9	25	1.2	0	7732072	5317255	10611312	46.991	2.40
10	25	0.8	10 mM	7516271	4917832	5911307	7.621	4.80

Food samples

In the first quarter of 2022, 66 samples of honey mixed herbal, mostly Ginseng and Tongkat Ali, sachets of different brands were randomly obtained from various pharmacies and drug stores in Amman, Jordan. The samples were stored in the dark at room temperature (20–25^o C). The samples were mixed at room temperature until a homogeneous solution was obtained.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The chromatographic conditions were optimized by investigating various mobile phase compositions, flow rate, and addition of buffer to obtain the best chromatogram separation in the shortest analysis time. Variations in the ratio of 0.1% formic acid in acetonitrile at a proportion of 25–45 % in the mobile phase, variation of flow rate from 0.8 to 1.2 mL/min, and ten mM ammonium formate were also investigated. Data were analyzed using Minitab® 20.0 software to maximize the peak area of PDE_5 inhibitors, optimize the resolution between vardenafil and sildenafil and decrease the retention time of tadalafil.

The present work sought to determine the combined effects of buffer, flow rate, and mobile phase composition on the reverse-phase liquid chromatographic behaviour of PDE_5 inhibitors. The effect of a buffer was also tested by adding ten mM ammonium formate to provide a maximum peak area of PDE_5 inhibitors and to decrease the retention time of tadalafil without affecting good separation resolution between the vardenafil and sildenafil peaks. Moreover, a flow rate between 0.8 to 1.2 mL/min was used to examine its potential impact on the resolution between vardenafil and sildenafil and the retention time of tadalafil. The DOE was applied to unearth the best suitable percentage of 0.1% formic acid in water to 0.1% formic acid in acetonitrile. Furthermore, adding ammonium formate as a buffer modifier at a concentration of 10 mM and finding the optimum flow rate to get the best-resolved peak area of vardenafil and sildenafil and decrease the retention of tadalafil. CCD with ten experimental points (Table 1) was performed randomly at all points. A quadratic polynomial model was used to fit the experimental data. Figures 1 and 2 depict the prediction profilers available on the response surface (Figures 1 and 2).

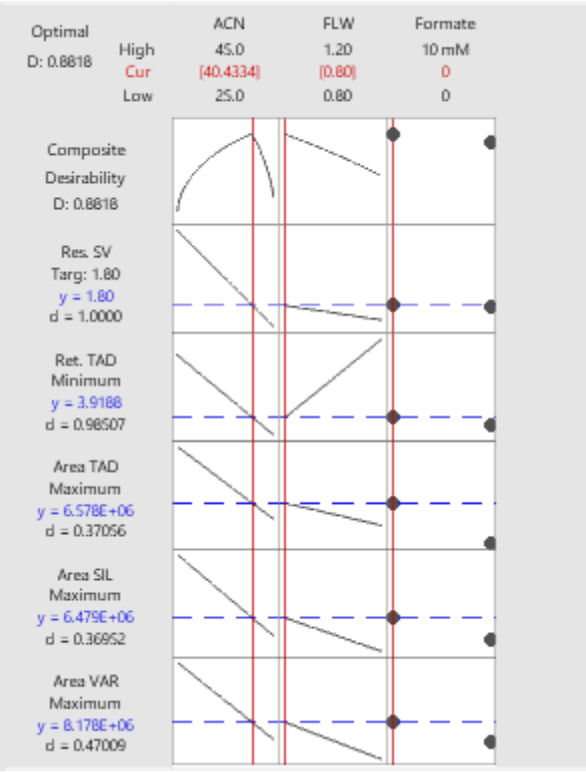
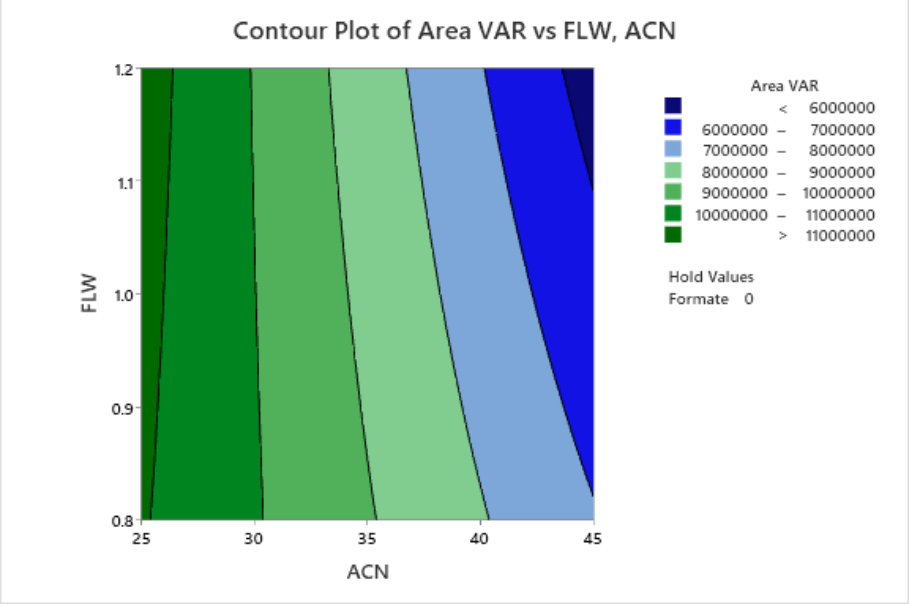
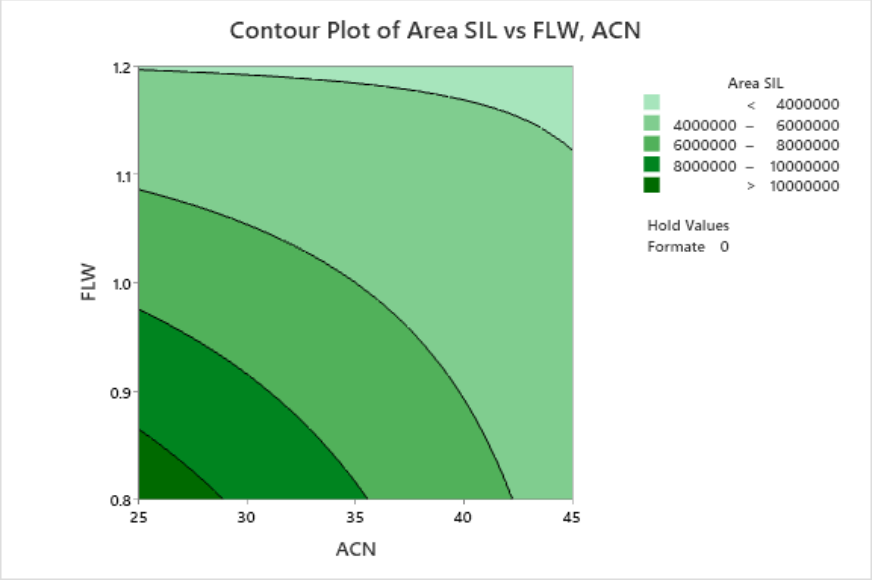


Figure 1: The Maximum Desirability Profiler displays optimal settings of flow-rate 0.8 and 0 mM ammonium formate using 60:40 (%, v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution. It gave 0.88 composite desirabilities of peak area of PDE_5 inhibitors, resolution between vardenafil and sildenafil and the retention time of tadalafil.

a



b



c

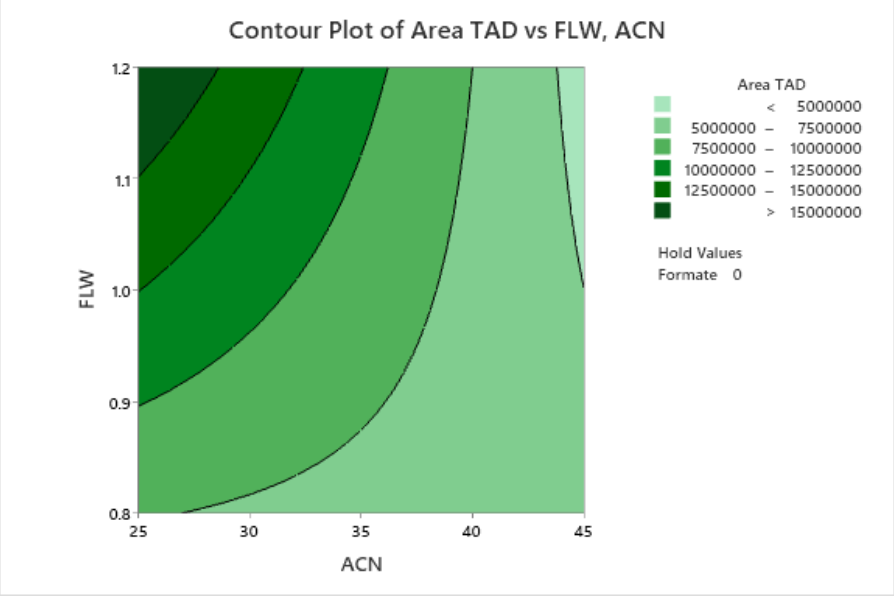


Figure 2: An overlay contour plot of both PDE5 inhibitors Vardenafil (a) and Sildenafil (b) peak area with 24 experimental points (the black dot point)

Figure 1 illustrates the Maximum Desirability Profiler and indicates optimal settings of flow-rate 0.8 and 0 mM ammonium formate using 60:40 (%, v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution. It gave 0.88 composite desirabilities of peak area of PDE_5 inhibitors, resolution between vardenafil and sildenafil, and comparatively short retention time of tadalafil.

Figure 2 indicates that the maximum peak area under the

curve can be achieved for PDE_5 inhibitors using 75:25 (v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution at different flow rates. However, this composition solution was avoided due to the broad peaks and comparatively long retention times when the water composition exceeded 70%. Additionally, using mobile phase composition of 55:45 (v/v) 0.1% formic acid in Water/ 0.1% formic acid in acetonitrile solution at 1.2 mL/ min, vardenafil, and sildenafil did not separate. Conversely, to the compositions above, a mobile phase composition of 60:40 (v/v) 0.1% formic acid in water/acetonitrile solution at a flow rate of 0.8 mL/min displayed optimal settings and offered adequate separation between vardenafil and sildenafil peaks with reasonable and acceptable retention times, as well as good peaks areas for all three PDE_5 inhibitors (Figure 3). The experimental data is in good agreement with the prediction of the Maximum Desirability Profiler.

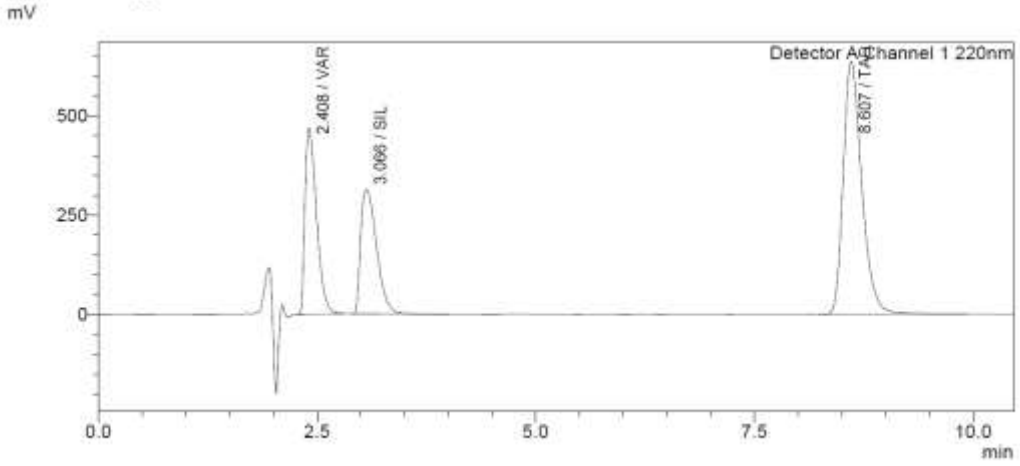


Figure 3: HPLC chromatogram of PDE5 inhibitors standard solutions containing 50 mg/L of vardenafil, sildenafil, and tadalafil with a retention time of 2.4, 3.0, and 8.6 minutes, respectively.

The bottleneck of any chromatographic system is the column, where the actual separation between analyte mixtures occurs. The column selection strongly relies upon the prior knowledge of the physical and chemical properties of the analyte and matrix [31]. The column type, length, and particle size were optimized by studying different HPLC columns under the optimized mobile phase conditions to obtain better chromatography in the shortest analysis time. The studied columns were:

A. Shimadzu Shim-pack GIST 5µm C8 150 x 4.6 (Japan),
B. Shimadzu Shim-pack GIST 5µm C18 150 x 4.6 (Japan),
C. Fortis Technologies 5µm Fortis C18 250 x 4.6 (Munich, Germany),
D. Agilent Technologies ZORBAX Eclipse Plus, C18, 3.5 µm, 4.6 x 150 mm (Santa Clara, USA).

Table 2 shows that the column significantly affects the retention time and sensitivity (Area %) of PDE_5 inhibitors. Column A (Shim-pack GIST 5µm C8) has a high sensitivity for sildenafil (97%) and tadalafil (96%) but suffers from less sensitivity for vardenafil (91%). On the other hand, column B (Shim-pack GIST 5µm C18) shows excellent sensitivity for vardenafil (100%) and high sensitivity for sildenafil (97%) and tadalafil (96%). Additionally, column B showed an optimal resolution (Rs ~ 1.93) between vardenafil and sildenafil and the second shortest retention time (less than 10 minutes) after column D.

Table 2: The HPLC column packing materials investigated (Vardenafil, Sildenafil, and Tadalafil at 260, 227 and 303 nm at mobile phases (60:40 (%, v/v)) flow rate mL/ min and column temperature 20°C.

Column		PDE_5 inhibitors	Retention Time (min)	Area %	Resolution
A	**Shimadzu Shim-pack GIST 5µm C8 150 x 4.6**	Vardenafil	2.466	91%	-
		Sildenafil	3.275	97%	3.106
		Tadalafil	10.307	96%	19.849
B	**Shimadzu Shim-pack GIST 5µm C18 150 x 4.6**	Vardenafil	1.934	100%	-
		Sildenafil	2.474	97%	2.586
		Tadalafil	9.615	96%	20.770
C	**Fortis Technologies 5µm Fortis C18 250 x 4.6**	Vardenafil	3.429	99%	-
		Sildenafil	4.667	100%	3.641
		Tadalafil	19.085	100%	19.666
D	**Agilent Technologies Zorbax Eclipse XDB-C18 150 mm × 4.6 mm**	Vardenafil	1.959	91%	-
		Sildenafil	2.510	93%	2.716
		Tadalafil	6.870	94%	16.315

The 25 cm columns (column C; 5µm Fortis C18 250 x 4.6) showed the highest sensitivity for all analytes; 99% for vardenafil and 100% for sildenafil and tadalafil, but was associated with a significant increase in retention times (~20 min).

The 3µm particle size column D showed good sensitivity for vardenafil (91%), sildenafil (93%), and tadalafil (94%). Furthermore, it had an optimum resolution (Rs ~ 1.96) between vardenafil and sildenafil, with the shortest retention time of fewer than 8 minutes. Ultimately, we chose column B as it had the highest sensitivity and the second shortest retention time. The optimized conditions resulted in an effective separation of the PDE_5 inhibitors in a run time of 10 min. The average retention time was 1.9 min for vardenafil, 2.5 min for sildenafil, and 9.6 min for tadalafil.

To assess whether the PDE_5 inhibitor mixed standards could be distinguished and well-separated from the interfering substances in the sample matrix, an adulterated honey-mixed herbal sample was pretreated using the modified sample preparation method and separated using a BDS 3µm C8 150 x 4.6. The chromatograms, which indicate the selectivity of the procedure, are shown in Figure 4. The chromatograms of the adulterated honey-mixed herbal sample showed that all peaks of the PDE_5 inhibitor standard are well separated from the interfering substances found in the matrix with reasonable retention times.

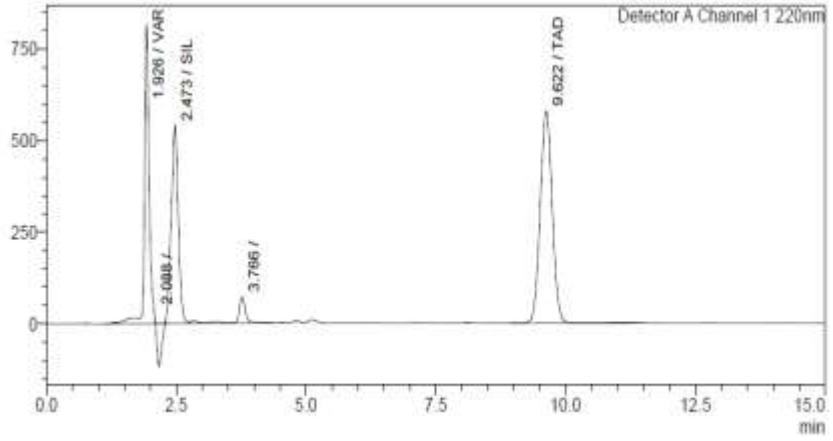


Figure 3: HPLC chromatogram of PDE_5 inhibitors standard solutions containing 50 mg/L of vardenafil, sildenafil, and tadalafil with a retention time of 2.4, 3.0, and 8.6 minutes, respectively.

Selection of detection λ-max

To choose the best wavelength, a spectrum of three PDE_5 inhibitors solution (50 mg/L) analyzed using the chosen HPLC mobile phase at a flow rate of 0.8 mL/min (Figure 3) was generated to optimize the detection of PDE_5 inhibitors. Based on a review of pertinent literature, we found that many articles analyzed sildenafil, vardenafil, and tadalafil at different wavelengths [24, 32]. Sildenafil was analyzed at λs of 225 [33], 230 [32, 34], and 292 [21, 33, 34], and vardenafil was analyzed at λs of 230 [32, 34], and 292 [21, 34], whereas tadalafil was analyzed at λs of 220 , 230 [32, 34], 283 [34] , 284 [24] and 292 [21, 34]. The optimization procedure for the wavelength selection was carried out by analyzing the targeted PDE_5 inhibitors at five different wavelengths (i.e., at λs of 220, 230, 245, 284, and 292) until the maximum sensitivity (peak area counts of sildenafil, vardenafil and tadalafil standards mixture) was obtained.

Table 3 shows substantial differences in the peak area counts for sildenafil, vardenafil, and tadalafil when applying different wavelengths. As the wavelength changed from 220 to 295 nm, the peak area for vardenafil, sildenafil, and tadalafil were substantially decreased, from 100% to 15%, 45%, and 24%, respectively. Therefore, a wavelength of 220 nm was selected to quantify sildenafil, vardenafil, and tadalafil in honey samples. Alternatively, the wavelength of 230 nm can be selected to avoid UV absorbance cut-off of the selected mobile phase for quantifying sildenafil, vardenafil, and tadalafil in honey samples as the peak area for sildenafil, and vardenafil, were slightly decreased, from 100% to 93%, 89%, and 72%, respectively (Table 3).

Table 3: The effects of the wavelength (nm) on the chromatographic peak areas percentage for PDE_5 inhibitors.

PDE_5 inhibitor	**Wavelength (nm)**								
	220	230	245	283	284	285	291	292	295
VAR	100%	89%	77%	22%	22%	21%	17%	21%	15%
SIL	100%	93%	66%	41%	41%	42%	45%	44%	45%
TAD	100%	72%	25%	29%	29%	35%	26%	26%	24%

Method validation

The method was validated internally regarding linearity, accuracy, intra-day and inter-day precision, the LOD, and the LOQ. The linearity was tested using a mixture of the three PDE_5 inhibitors standards in a concentration range from 12.5 to 200 mg/L for sildenafil, vardenafil, and tadalafil.

Table 4 shows good linear relationships between the concentration of the analyte and the peak response, with correlation coefficients more significant than 0.9999 for all analytes. Calibrations with ICH standard solutions were utilized for quantitation of PDE5 inhibitors, and Statistical analysis was performed using SPSS program version 19. Observed differences between groups were compared using analysis of variance (one-way ANOVA). Differences were regarded as significant, with $P \leq 0.05$.

Table 4: Linearity range, Equation, r^2 value, LOD and LOQ of sildenafil, vardenafil, and tadalafil.

PDE_5 inhibitor	**Linearity Range** (mg/L)	**Equation**	**r^2**	**LOD** (mg/L)	**LOQ** (mg/L)
Sildenafil	12.5 – 200.0	y = 33306x - 64947	0.9999	1.70	5.65
Vardenafil	12.5 – 200.0	y = 30981x - 17137	0.9999	2.16	7.21
Tadalafil	12.5 – 200.0	y = 70515x - 21690	0.9999	1.03	3.42

The accuracy was calculated by determining the recoveries of PDE_5 inhibitors from honey samples at concentrations of 50, 100, and 150 mg/L of sildenafil, vardenafil, and tadalafil standards; the spiked samples were analyzed in triplicates (Table 5) and calculated using the following formula: [35]:

$$\text{Recovery (\%)} = \frac{\text{Recovered Amount (mg/L)}}{\text{Added Amount (mg/L)}} \times 100$$

Table 5: Mean of recoveries and RSDs (n=5) of sildenafil, vardenafil, and tadalafil spiked into clean Honey-Mixed Herbal Sachets and Honey Sachets samples at three spiking levels using HPLC method (n = 3)[a]

PDE_5 inhibitor	Spiking Level (mg/L)	Honey-Mixed Herbal Sachets	Honey Sachets
		Mean of Recovery (%) ± RSD (%)	
Sildenafil	50	94.1±0.8	93.0±0.7
	100	103.3±0.3	98.6±0.8
	200	101.2±0.6	100.1±0.8
Vardenafil	50	91.4±1.2	96.0±0.4
	100	102.7±0.6	101.2±0.1
	200	101.2±0.7	100.5±0.3
Tadalafil	50	95.7±0.4	96.0±1.1
	100	103.2±0.7	100.2±0.5
	200	101.0±0.3	102.5±0.3

[a] n: is the number of replicates

The recovery percentages ranged from 93.0% to 103.3%, with an RSD of less than 2%.

The sensitivity was determined by assessing the LOD and LOQ. LODs and LOQs were determined experimentally as the lowest concentration giving a response of three- and six times to the baseline noise, respectively. The LOD of sildenafil acid, vardenafil acid, and tadalafil was 1.70, 2.16, and 1.03 mg/L, respectively. The LOQs were 5.65, 7.21, and 3.42 mg/L, respectively (Table 4). Or Table 5

Intra-day precision was calculated by assaying five replicates of the same sample at a spiked level of 50 mg/L of sildenafil acid, vardenafil acid, and tadalafil on the same day. For the inter-day precision, five replicates of the same sample at a spiked level of 50 mg/L of sildenafil acid, vardenafil acid, and tadalafil were analyzed on three consecutive days. The intra-day precision and inter-day precision were calculated and tabulated in Table 6. The intra-day precision (n= 5) values were between 0.4 and 1.2%, whereas the inter-day variation (n= 15) values were between 1.0-1.7%. The complete separation of the peaks, the low RSD, LOD, and LOQ data obtained from this work compared with reported values confirm this method's good reproducibility and repeatability.

Table 6: The intra-day precision and inter-day precision of sildenafil, vardenafil, and tadalafil expressed as RSD% values

PDE_5 inhibitor	**Spiking Level** (mg/L)	**Intra-Day Precision** (n = 5)[a]	**Inter-Day Precision** (n = 15)[a]
Sildenafil	50	0.8	1.1
Vardenafil	50	1.2	1.7
Tadalafil	50	0.4	1.0

[a] n: number of replicates

Considering the data obtained from the method validation, the current HPLC–UV analysis measured with the aid of response surface methodology, experimental design, and sample preparation procedures is considered a selective, precise, and robust method to determine sildenafil acid, vardenafil acid, and tadalafil PDE_5 inhibitors in honey samples.

Food samples analysis

The developed method was applied to analyze the targeted PDE_5 inhibitors in 66 samples of honey mixed herbal sachets declared as purely 'natural' erectile supporters, which were collected in the first quarter of 2022 from various community pharmacies in the capital city of Amman. The collection method was based on approved international methods, where the samples were preserved and transported to the laboratory in conditions commensurate with the storage methods. In addition, all information related to each sample was recorded in a sample record, including the type of sample, its source, and the date taken to interpret and evaluate the results. The samples were distributed as follows: 8 samples of local origin, 28 samples from the United States of America and Canada, 30 samples from European countries, and finally, 18 samples from Asia.

The analysis showed that eight samples turned out to be positive for non-declared PDE_5 inhibitors. Sildenafil was considered to be the primary active compound to be detected (1.1 mg/sachet [local samples] to 124 mg/sachet [mixed herbal sachets from Asia]). In contrast, tadalafil was only detected in five samples ranging from 0.67 mg/sachet [local samples] to 76.6 mg/sachet [mixed herbal sachets from Europe]. On the other hand, vardenafil was not detected in any of the tested samples from all sources. It is worth mentioning that samples of the same commercial brand tested positive for the PDE5 inhibitors with varying concentrations in each sachet. In contrast, others tested negative, indicating no systemic way of fortifying the PDE5 inhibitors and lack or absence of quality control and quality assurance procedures. This is a catastrophic and lethal error as the consumer might take a multi-sachet dose, mainly that all obtained honey-mixed herbal sachets samples lacked patient leaflet with some dare to claim it is safe for use by printing a legal Disclaimer as below:

The product does not cause any side effects and is safe for men of all ages; however, it is not to be used by people with kidney failure, heart disease, chronic hypertension, ischemia, and children under 18 years old.

Patients over 40 with chronic diseases such as hypertension, ischemic heart disease, diabetes, depression, and atherosclerosis generally have ED as a complication. In recent years, herbal products are becoming more popular than ever as an alternative to prescription drugs for numerous reasons, including a common belief that they are safe, obtaining herbal products without a prescription or seeing a doctor or even a pharmacist, and the myth belief of folklore tradition that these herbs work better than prescribed drugs in the market. However, our findings are in-line with all previous observations, which further emphasize that herbal remedies are frequently being adulterated with undeclared synthetic drugs to achieve the desired action, and this may inevitably be associated with life-threatening ramifications on its own or as a result of clinically significant drug-drug interactions [18, 19, 21, 24].

CONCLUSION

The importance of this work paves the foundations for quickly and effortlessly performing a simple analytical method to determine and quantify the presence of PDE_5 inhibitors in honey-mixed herbal sachets. This is paramount as commercial fraud in such products might lead to fatalities or severe side effects. The findings of this project revealed astonishing and alarming information about these products, such as the random fortification quantity of PDE5 inhibitors and the failure to pronounce the existence of such materials on the product's packaging. Moreover, a simple, rapid, inexpensive, and effective sample preparation method has been developed to determine vardenafil, sildenafil, and tadalafil in honey-mixed herbal sachets in the hope of encountering and combating commercial frauds and ultimately saving lives. The sensitivity of the HPLC–UV instrument could be significantly enhanced by optimizing the mobile phase composition and the type, length, and particle size of the HPLC column. The developed sample preparation procedure is based on a single extraction step without employing pre-treatment processes and thus can be recommended as an alternative to the time-consuming precipitating step, steam distillation multiple steps, or solid-phase extractions clean up a step for PDE_5 inhibitors determination in food. A C18 150 mm × 4.6 mm x 5 µm column at 20^{o}C performed separation of the vardenafil, sildenafil, and tadalafil with higher selectivity and sensitivity and within shortened retention time. Excellent linearity and repeatability, high recoveries, , and reproducibility values were obtained, indicating the developed method's suitability for determining PDE_5 inhibitors in Honey-Mixed Herbal Sachets. Compartively short retention times for sildenafil acid, vardenafil acid, and tadalafil were found 1.93, 2.47, and 9.62, respectively, while the limits of detection (LOD) were 1.70, 2.16, and 1.03 mg/L, and the limits of quantification (LOQ) were 5.65, 7.21, and 3.42 mg/L.

Acknowledgments

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تحسين والتحقق من طريقة HPLC-UV لتحديد Vardenafil وSildenafil وTadalafil في أكياس العشبية المصنوعة من العسل باستخدام تصميم التجربة

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ملخص

تم تطوير طريقة لتحديد وتحليل متزامن للسيلدينافيل، فاردنافيل، وتادالافيل في أكياس عشبية مخلوطة بالعسل باستخدام كروماتوجرافيا سائلة عالية الأداء مع كاشف الأشعة فوق البنفسجية (.HPLC-UVهذه الطريقة تلغي استخدام الإجراءات المعقدة وتلغي عمليات المعالجة المسبقة التي تستغرق وقتًا طويلاً وعملا كثيفا. في غضون عشر دقائق (عند 25 درجة مئوية) تمت عملية الفصل بنجاح لكل من سيلدينافيل و فاردينافيل و تادالافيل باستخدام عمود الفصل من نوع C18 GIST Shim-Pack الطول 150 ملم، القطر الداخلي 4.6 ملم، قطر الحبيبات 5 ميكروميتر) مع انتقائية وحساسية عالية. كانت تركيبات الطور المتحرك عبارة عن خليط بنسبة 60:40 (حجم لحجم) من حمض الفورميك 0.1 % المذاب في الماء وحمض الفورميك 0.1 % المذاب في الأسيتونيتريل. باستخدام الطور المتحرك كخليط استخراج، أعطت معدلات استرداد في نطاق 93.0–103.3٪ عند تراكيز من 50–150 ملغم / كغم مع الانحرافات المعيارية النسبية (RSDs) أقل من 10٪. وكانت النتائج الدقيقة داخل اليوم وبين اليوم في حدود 0.4–0.8 ٪ و1.0–1.7 ٪. علاوة على ذلك، كانت زمن انحباس ل السيلدينافيل، الفردنافيل، وتادالافيل 1.93، 2.47 و 9.62 دقيقة، على التوالي، و لقد كان الحد الأدنى للقياس النوعي 1.70 (LOD)، 2.16 و 1.03 ملغم/لتر، في حين أن الحد الأدنى للقياس الكمي (LOQ) كانت 5.65 و 7.21 و 3.42 ملغم/لتر.

الكلمات الدالة: HPLC، تصميم التجربة، فاردينافيل، سيلدينافيل، وتادالافيل، مثبط PDE5.

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Evaluation of Changes in the Ganglionic Cell inner Plexiform Layer and Macular Retinal Nerve Fiber Layer in Patients Receiving Hydroxychloroquine

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ABSTRACT

Backgrounds: To evaluate changes in the thickness of ganglion cell-inner plexiform layer and macular retinal nerve fiber layer using ocular coherence tomography in patients exposed to hydroxychloroquine .

Methods: This was a retrospective, cross-sectional study of patients on hydroxychloroquine therapy. Ocular coherence tomography images showing ganglion cell-inner plexiform cell layer and macular retinal nerve fiber layer thickness were obtained and compared to controls. The relationship between the thickness of ganglion cell-inner plexiform and macular retinal nerve fiber layer, duration and cumulative dose of hydroxychloroquine were evaluated.

Results: In all, 219 subjects were included. The Thickness of the ganglion cell-inner plexiform thickness was significantly less than controls (p = 0.006). The average macular RNFL thickness was less in the study compared to the control groups, but not statistically significant (p = 0.389). There was no significant correlation between ganglionic cell-inner plexiform and macular retinal nerve fiber layer with duration, daily dose, or cumulative dose of hydroxychloroquine.

Conclusion: Thinning of the ganglionic cell- inner plexiform layer could be an early indicator of retinal toxicity before the appearance of clinical retinopathy.

Keywords: Hydroxychloroquine, ganglion cell-inner plexiform layer, macular retinal nerve fiber layer, spectral-domain optical coherence tomography.

INTRODUCTION

Hydroxychloroquine (HCQ) is a well known treatment option for many rheumatological and autoimmune disorders like systemic lupus erythematosus and rheumatoid arthritis[1] and other diseases like malaria[2]. However, many side effects were reported. Deposits on the cornea, ciliary body, retina, and the choroid are the classical form of ocular side effects [3,4]. Early discontinuation of the medication may reverse most of the side effcts [1]. However, Bulls eye maculopathy is an irreversible form of retinal toxicity which affects the macula and leads to loss of central visual field, loss of visual acuity and color vision defects[1]. Animal studies confirmed that toxicity results in in damage to perifoveal retinal ganglion cell[5] . Early detection of toxicity before it is clinically visible on fundus examination is important, because immediate discontinuation of HCQ might reverse early retinal toxicity[3]. Many investigations are conducted to detect early changes such as visual field test, ocular coherence tomography, and electroretinogram (ERG)[3].

HCQ induced retinal toxicity was considered a rare condition; but higher incidence has been reported in a recent study than it was previously known and is dose dependent[5].

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The toxcity rate can reach up to 1% [2]. Furthermore, worsening of retinopathy may happen even after discontinuation of HCQ [1]. Toxicity can be detected by subjective tests like measurement of visual acuity , slit lamp examination, dilated fundus examination and automated central perimetry 10-2 test [5,6]. Useful objective tests are fundus autofluorescence photography, multifocal ERG, and spectral domain OCT (SD-OCT) macular measurements [7]. Screening recommendation by the Royal college of Ophthalmologist is to perform a colored fundus photograph along with a SD OCT, ideally within the first 6 months of treatment. If the patient has a normal examination with no additional risk factors screening tests should be performed after 5 years and theannuallyly. However, if the patient has at least one risk factor screening tests should be done after one year. Risk factors that are considered significant are: concamitant use of Tamoxefin , impaired renal function test, a daily dose of more than 5 mg/kg/day and chloroquine use[8]. In Jordan, these recommendations are adopted [8,9]. the Retinal toxicity should be demonstrated on two screening tests , being objective , before discontinuation of treatment in patients suspected to have HCQ-induced retinal toxicity[10,11].

The aim of this study was to evaluate the thickness of ganglion cell inner plexiform layer (GC-IPL) and that of the macular retinal nerve fiber layer (RNFL) in patients on HCQ therapy and to compare these values with those of age-matched controls. This might serve as a tool to detect early macular retinal changes before the changes are clinically visible.

METHODS:

The study was approved by the Institutional Review Board of the University of Jordan number 19/20/279 and written informed consent was obtained from every participant in the study. It was performed in accordance with the Helsinki Declaration of 1964, and its later amendment.

This was a retrospective cross-sectional study. Patients on HCQ therapy who visited Jordan University Hospital between September 2018 and September 2019 and who were taking the medication for systemic lupus erythrematousus were included. All participants data who underwent a standard ophthalmic examination including measurement of GL-IPL and macular RNFL by OCT extracted. Thickness of GI-IPL and macular RNFL of age-matched healthy subjects were measured and compared. Patients on HCQ therapy and those in the control group underwent a complete ophthalmic examination including best corrected visual acuity, intraocular pressure measurement, dilated fundus exam, color examination using Ishihara pseudoisochromatic plates (Kanehara Shuppan Co. Ltd., Tokyo Japan) and OCT.

The inclusion criteria were: best corrected visual acuity$\geq$ 0.8, normal ophthalmic examination, intraocular pressure at baseline and at any ophthalmic visit of < 21 mmHg,normal appearance of the optic disk and the retina.

Exclusion criteria were as follows: any ocular or systemic disease that may affect the retina or the optic nerve excluding systemic lupus erythematosus for which participants were receiving the drug, history of ocular surgery, history of eye trauma, ocular inflammation, spherical equivalent more than 6 diopters, optic disc or retinal disease, or optic disc anomaly or glaucoma. Any patient with media opacity that interfered with the quality of the OCT was also excluded.

To measure the macular thickness, an 8x8 mm macular area centered around the foveal thickness was recorded using the OPTOPOL SD OCT machine (SD OCT version 7.2.0, OPTOPOL Technology Sp. z.o.o., Poland). The central foveal and average macular thickness were recorded. The OCT machine differentiated between the retinal tissue interfaces and detected the thickness of GC-IPL and RNFL. RNFL thickness was measured from the inner margin of the internal limiting membrane to the outer margin of the RNFL layer after being automatically segmented using OCT. all the images were revised and manual resetting was carried for any artifact. A machine

tracking system was used to compensate for any eye movement.

Statistical analysis:

We used SPSS version 21.0 (Chicago, USA) for analysis. Continuous variables like age were described as mean (± standard deviation). Other nominal variables (e.g., gender) were described as count (frequency). We performed an independent sample t-test to analyze the difference in the mean values between cases and controls and between mean difference in age between the groups. Paired-sample t-tests were used to analyze the mean differences in OCT measurements at baseline and at follow-up in both groups, using the filter function to isolate the study subjects and controls for the test. Data is presented as means (95% confidence interval [CI]). The chi-square test was used to compare the frequency of gender and other risk factors between the groups. Pearson correlation coefficient was used to analyze the correlation between the OCT findings, duration of treatment, and cumulative dose of HCQ. The correlation between the thickness of GL-IPL and macular RNFL, and daily and cumulative HCQ doses were analyzed. All underlying assumptions were met unless otherwise indicated. Ap value of less than 0.05 was considered as statistically significant.

RESULTS

In all, 219 participants were included in this study with a mean age of 43.38. ±17.39. years. The study group comprised100 (20 male and 80 female) patients with a mean age of 45.28 (± 12.24) years, and the control group comprised119 patients (44 males and 75 females), with a mean age of 41.79 (± 20.67) years. There was no significant difference in age, gender or spherical equivalent between the groups (p= 0.123). The mean cumulative dose of HCQ was 5666.4± 4329.0 g, and the mean duration of HCQ therapy was 56.5 ± 36.3 months. The mean daily dose was 313.9 ± 96.9 mg. The cumulative dose was measured by multiplying the daily dose by the number of days in which the participant used the drug. The participants' demographic data are presented in table 1 and HCQ treatment data in table 2.

Table 1. Description of the - overall demographic characteristics of the study population

	N	Minimum	Maximum	Mean	Std. Deviation
Age	219	9.0	91.0	43.384	17.3852
RNFL thickness(um)	219	22.0	38.0	28.9	2.6
GCL+IPL thickness(um)	219	50.0	110.0	87.37	7.2

Table 2. Shows HCQ treatment data

	N	Minimum	Maximum	Mean	Std. Deviation
Duration of HCQ therapy (months)	100	6.0	168.0	56.5	36.3
Accumulative dose in grams	100	36000.0	2016000.0	566640.0	432896.6
Average daily dose in grams	100	100.0	400.0	314.0	96.9

The average GCL-IPL thickness was 85.9 ± 8 μm (Range: 50 - 98) in patients on HCQ therapy compared to 88.6 ± 6.2 μm (Range: 75 - 110) in the control group (p=0.006) The average RNFL thickness was 28.2 ± 2.8 μm (range: 22 - 35) in patients on HCQ therapy and 29.2±2.5 μm (range: 23 - 38) in) in the control group (P>0.05).

There was a significant difference in the mean GCL-IPL thickness between the groups (p = 0.006), with a mean

difference of 0.31 μm (95% confidence interval ranging from 0.79 to 4.56); the mean thickness was higher in the control group. However, the average macular RNFL thickness was statistically similar in both groups (p = 0.389).Table 3 shows details of the differences between the groups.

Table 3. Shows details of the differences between the groups.

	Group	Mean thickness (µm)	Std. Deviation	p value
RNFL thickness	HQC group	28.160	2.8203	0.389
	Control group	29.180	2.5165	
GCL+IPL thickness	HCQ group	85.910	7.9647	0.006
	Control group	88.588	6.1869	

There was no significant correlation between GCL+IPL and macular RNF thickness with either daily dose (p = 0.229) or the cumulative dose (p = 0.678), as demonstrated in Table 4.

Table 5 shows the correlations between SD-OCT measurements (average RGC-IPL and RNFL thickness) and mean age of patients in both groups. There was a statistically significant negative relationship between age and average GC-IPL thickness in controls ($p < 0.05$) but not in patients on HCQ therapy . There was no statistically significant correlation between the age of patients and average macular RNFL thickness in control group ($p > 0.05$). However, a significant positive correlation in RNFL was found in patients receiving HCQ (P=0.013)

Table 4. Correlations between measured thicknesses and cumulative and daily HCQ dose, and duration of HCQ therapy

	Cumulative dose		Daily dose		Duration of therapy	
	R	p*	R	p*	R	p*
Avarage RGC-IPL th	-0.042	0.678	-0.121	0.229	-0.046	0.649
Avarage RNFL	-0.139	0.168	-0.087	0.389	-0.175	0.081

* Pearson's correlation test; RGC-IPL: Retinal ganglion cell-inner plexiform layer; RNFL: Retinal nerve fiber layer

Table 5. Correlations between Age of both groups and Retinal ganglion cell-inner plexiform layer thickness and Retinal nerve fiber layer thickness

	Group 1 patients on HCQs		Group 2 Controls	
	R	p*	R	p*
Average GCL-IPL	0.000	0.678	-0.241	0.008
Avarage RNFL	0.248	0.013	0.069	0.453

* Pearson's correlation test; GCL-IPL: Retinal ganglion cell-inner plexiform layer; RNFL: Retinal nerve fiber layer

DISCUSSION

Our study shows that the GC-IPL layer was significantly thinner in patients receiving HCQ compared to controls ,thinning of the macular RNF was also detected in patients receiving hydroxychloroquiene but was not statistically significant . However, the thickness of the GC-IPL and macular RNFL were not negatively related to the duration and cumulative dose of HCQ. Our study is the

first study to be carried on a Middle Eastern population with large sample size and longer duration.

Retinopathy secondary to HCQs is associated with macular retinal pigmentary changes[6]. It was proposed that HCQ toxicity early alters the ganglionic cells with possible secondary macular RNFL thinning and damage[2,3]. The exact way of HCQ toxicity is not fully understood. At the first stages , the drug accumulates in the cytoplasm of retinal ganglion cells which leads to degeneration of the ganglion cells. Later on, degeneration of the photoreceptors and retinal pigment epithelium result from binding to melanin[12,13] with resultant visual field loss, decreased visual acuity and impaired color vision[5,7] Animal studies showed that accumulation of HCQ in the retinal ganglionic cells may affect the photoreceptors long before the RPE shows signs of toxicity [14]. SD- OCT imaging showed loss of the parafoveal photoreceptor inner segment – outer segment junction and thinning of the outer nuclear layer in patients receiving HCQ, as a result retinal thinning may be an early indicator of retinal toxicity.[15,16,17] . It was demonstrated that SD-OCT images might indicate retinal toxicity long before occurrence of visual field loss [11,12].

Until recently, there are few studies with limited sample sizes, using variable imaging techniques, different treatment regimens and short duration of follow up , moreover, the results are variable and conflicting [11]. Most previous studies investigated pathologies associated with the outer retinal segment, involving the retinal pigment epithelium and photoreceptors[9],other studies have demonstrated HCQ-associated damage in the inner retinal segment only in normal-looking retinae [11]; however,damage was also observed in the inner and outer retina when abnormal fundus was clinically evident [14]. Few studies have evaluated the thickness of the GP-IPL layer, which is part of the inner retina; unfortunately, the number of subjects enrolled was small and the duration of HCQ therapy was short [15,16,18]. In the study conducted by Bulut et al demonstrated that retinal ganglion cell-inner plexiform layer of patients receiving hydroxychloroquine was statistically thinner than controls, which is consistent with our results, however, their totat number was small , the follow up duration was shorter and the sample was composed of females only [15].

Pasadhika S et al in their work demonstrated selective thinning of the macular inner retinae in the absence of clinically apparent fundus changes,[15] which is consistent with our finding as macular RNF and GC-IPL are parts of the inner layer. In our work the inner retina was further segmented into macualr RNFL and GC-IPL and we concluded that significant thinning was evident in the GC-IPL rather than in the macular RNFL.

Moreover, inconsistent results were found regarding the relationship between GC-IPL thickness, and cumulative dose and duration of HCQ therapy. While a group of investigators demonstrated that dose and duration of HCQ correlate negatively with average GC-IPL thickness [1], another study demonstrated no significant association [7], which is consistent with our results. It was postulated that retinal ganglionic cells show changes as early as first week after starting treatment with HCQ therapy and in photoreceptors soon afterward [8]. This might explain our findings that changes in the GC-IPL were not correlated with the duration of treatment or cumulative dose of HCQ.

The findings of this study are consistent with those reported in similar study recently conducted by Lee et al. [7]. It was observed that macualr RNFL thinning develop after clinically visible HCQ retinopathy [13,14]. In our cohort , all had normal looking retinae which may explain why patients on HCQ didn't have significantly thinner macular RNFL compared to controls.

However, paradoxical thickening of the macular RNFL was observed, compared to controls. Thickening of the RNFL was observed in many ocular pathologies such as retinitis pigmentosa and drug-related changes. It was explained that it is a chronic reactive change, secondary to retinal ganglionic cell stroma and axonal degeneration [16,17]

The sample size in our study is larger than that in previous works, and the control group had no previous ocular factors that might affect the quality of the OCT scan

and all the study population had the same indication for HCQ treatmemt and was composed of males and females.

Our study has several limitations. First, is the retrospective nature and the small sample size, which was due to the low incidence of patients receiving HCQ therapy. Second, the control group consisted of healthy subjects. The ideal group would be an age-matched group with the same rheumatological disorders but not receiving HCQ therapy. This is difficult as it is not always possible to perform ocular examinations in patients with rheumatological disorders without any concerned reasons. Third, the cross-sectional nature of the study made it difficult to evaluate the longitudinal effect of the medication. Further prospective studies with long-term changes are required to confirm our findings and to investigate if thinning of the GC-IPL precedes retinopathy.

In conclusion, macular GC-IPL showed significant thinning in patients on HCQ therapy but did not correlate with the duration and cumulative dose. Therefore, GC-IPL might serve as early biomarkers for HCQ toxicity. We suggest measuring GC-IPL thickness as an objective tool for early detection of HCQ induced toxicity using SD-OCT before it appeared clinically.

List of Abreviatoinns :

OCT: ocular coherence tomography

GC-IPL : ganglionic cell- inner plexiform cell layer

RNFL: retinal nerve fiber layer

ERG: and electroretinogram

SD-OCT: spectral domain ocular coherence tomography.

Declarations :

Ethics approval and consent to participate: The study was approved by the Institutional Review Board of the University of Jordan number 19/20/279 and written informed consent was obtained from every participant in the study. The study was performed in accordance with the Helsinki Declaration of 1964, and its later amendment

Consent to publish: all authors: all participants give their consent to publish the manuscript

Availability of Data and materials: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

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تقييم سماكة االطبقة العنقودية والطبقة الداخلية الضفيرة وطبقة الالياف الشبكية عند الذين يتناولون عقار الهيدروكسي كلوروكوين

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ملخص

الخلفية: لمعرفة التغيرات التي تحدث باستخدام جهاز التصوير الطبقي للشبكية عند المرضى الذين يستخدمون عقار الهيدروكسي كلوروكوين.

الطريقة: دراسة رجعية مقطعية للمرضى اذين يستخدمون عقار الهيدروكسي كلوروكوين. من خلال جهاز التصوير الطبقي للشبكية، تم الحصول وتحليل صور للطبقة العنقودية والطبقة الداخلية الضفيرة وطبقو الالياف الشيكية ومقارنتها مع الناس الاصحاء. تم دراسة العلاقة بين سماكة هذه الطبقات والمدة الزمنية للعلاج والجرعة الكلية.

النتائج : كان هناك 219 مشارك، كان معدل أعمارهم سنة 43.38 (±17.39). كان هناك 100 شخص من الذين يناولون العقار وكان معدت اعمارهم 45.28 +−(± 12.24) سنة وكان هناك 119 مشارك من مجموعة التحكم معدل اعمارهم 41.79(± 20.67) بدون وجود فارق عمري بينهم . كان هناك فرق بين سماكة الطبقة العنقودية والضفيرة الداخلية بين المجموعنين . ,(p = 0.006) (88.6+/-6 µm) vs. (85.6+/- 8 µm) معدل سماكة الخلايا الشبكية كانت أقل عند الذين يأخذون العقار بالمقارنة مع الأصحاء، 28.2±2.5 µm (range: 23 – 38) and 29.2±2.8 µm (range: 22 – 35) بالترتيب، لكن الفرق لم يكن ذو اهمية احصائية. لم يكن هناك علاقة بين سماكة الخلايا العنقودية والضفيرة الداخلية والالياف الشبكية المركزية مع مدة العلاج، الجرعة اليومية والرعة الكلية

الاستنتاج: سماكة الخلايا العنقودية والضفيرة الداخلية أقل عند الذين يأخذون عقار الهيدروكسي كلوركوين مقارنة مع الاصحاء. سماكة هذه الطبقة ممكن ان تستخدم كدليل مبكر لوجود سمية من هذا العقار قيل ان تظهر علامات سريرية.

الكلمات الدالة: هيدروكسي كلوركوين، الطبقة العنقودية والضفيرية الداخلية، الالياف الشبكية المركزية، التصوير الطبقي للشبكية.

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الناشر

الجامعة الأردنية
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عمادة البحث العلمي

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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