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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 pharmacoeconomics, pharmaceutical marketing, and management. Intellectual property rights for pharmaceuticals, regulations and legislations are also interesting topics welcomed from our colleagues in Schools of Law.

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

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

Volume 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

Dear Esteemed Researchers

As a member of the esteemed editorial board of the Jordan Journal of Pharmaceutical Sciences (JJPS), it is with great enthusiasm that I extend a cordial invitation to all dedicated researchers and practitioners in the pharmaceutical arena. At the heart of our commitment to advancing scholarly dialogue, JJPS stands as a beacon of excellence, indexed in Scopus (Elsevier), Ulrich's Periodicals Directory, Google Scholar, and EBSCO.

This upcoming volume heralds a unique opportunity for researchers to contribute to our journal's rich tapestry of knowledge. We eagerly seek submissions from those engaged in the formulation and exploration of innovative drug delivery systems, as well as review articles spanning multiple interdisciplinary fields within pharmaceutical sciences. JJPS serves as the nexus for groundbreaking research, offering a platform where diverse ideas converge to shape the future of pharmaceutical innovation.

Our dedication to sustainability is evident in our encouragement of submissions that delve into green and eco-friendly solvents and drug delivery systems. Recognizing the pressing need for responsible practices in the pharmaceutical industry, we aim to highlight research that champions environmentally conscious approaches, contributing to a more sustainable future.

Furthermore, we invite researchers to explore the realms of cost-effective innovation with new drug delivery systems for approved drugs. By focusing on reducing the costs associated with developing a new chemical entity, we collectively contribute to fostering accessibility and affordability in pharmaceutical advancements.

Publishing with JJPS not only ensures rigorous peer review and adherence to the highest standards of academic excellence but also provides unparalleled visibility on the global stage through our diverse indexing platforms. Your contributions will become integral to the ongoing dialogue that shapes the trajectory of pharmaceutical sciences.

Together, let us forge new frontiers in pharmaceutical research, innovation, and sustainability. Submit your work, and let your research be the cornerstone of positive change in our dynamic field.

Sincerely,

Professor Faisal Al-Akayleh
Editorial Board Jordan Journal of Pharmaceutical Sciences
Department of Pharmaceutics and Pharmaceutical Sciences
University of Petra
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CONTENTS

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Distribution of Vitamin D Status in a Group from Syrian Society

Talleh Almelli [1*]

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ABSTRACT

Objective: The aim of this work is to study the serum levels of 25-hydroxyvitamin D3 in a sample of healthy Syrians in the city of Homs.

Method: A cross-sectional study, including 690 ostensibly healthy participants, was conducted at the National Hospital of Homs. Serum levels of 25-hydroxyvitamin D3 were measured using chemiluminescent immunoassay.

Results: The overall prevalence of vitamin D inadequacy (insufficiency, deficiency, and severe deficiency) in the study samples was 76.5%. Additionally, 49% of the samples had vitamin D deficiency, with 18.5% suffering from severe deficiency. Furthermore, levels of 25-hydroxyvitamin D3 in females were lower than in males (11.3±2.3 ng/ml versus 39.6±11.28 ng/ml, respectively, $p < 0.0001$). Veiled women had serum levels of vitamin D lower than non-veiled women, 11.3±2.5 ng/ml versus 25.5±3.2 ng/ml, respectively, $p < 0.0001$. Female gender and clothing style were identified as independent risk factors for vitamin D deficiency.

Conclusion: The prevalence of vitamin D deficiency was very common in the study population, despite the sunny weather in Homs city most of the year. Further studies with larger groups, including other Syrian governorates, are needed to elucidate lifestyle and sociocultural behavior risk factors for vitamin D deficiency.

Keywords: 25-hydroxyvitamin D3, vitamin D deficiency, vitamin D insufficiency, sunlight, Syria.

1. INTRODUCTION

Vitamin D deficiency is one of the most significant public health problems affecting various age groups, including men, women, pregnant women, newborns, children, adolescents, adults, and the elderly, even in countries with ample exposure to sunlight throughout the year. Interestingly, the Middle East, a region that experiences sunlight most days of the year, has recorded the lowest levels of vitamin D, especially among women. This global health issue, linked to malnutrition and inadequate sunlight exposure, threatens millions of people [1,2].

Vitamin D, a fat-soluble vitamin and considered a hormone precursor, plays a crucial role in bone metabolism. It controls calcium absorption, mediates bone mineralization with parathyroid hormone, maintains the internal stability of calcium and phosphorus, and contributes to various physiological and metabolic functions [3,4]. Vitamin D exists naturally in two biological forms: vitamin D2 ('ergocalciferol') in plant sterol and vitamin D3 ('cholecalciferol') in fish oil, produced after skin exposure to short ultraviolet light (UVB). UVB converts pro-vitamin D3 in the skin to vitamin D3, which is then transformed in the liver to 25-hydroxyvitamin D3 (25(OH) D3) and further converted in the kidney into the active form, 1,25 di-hydroxy vitamin D3. The final form can bind to nuclear receptors in target tissues, regulating target genes [5].

Vitamin D receptors are present in various body tissues, and studies have indicated that vitamin D deficiency is associated with autoimmune, neoplastic, and metabolic diseases [6,7,8]. Insufficient nutritional resources and the

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chronic use of certain medications that stimulate vitamin D metabolism are common causes of vitamin D deficiency.

Recently, several studies have revealed a significant association between vitamin D deficiency and the high risk of infection, hospitalization, and increased mortality rate of COVID-19 [9,10,11]. This suggests a key role for vitamin D supplementation in the treatment and/or prevention of COVID-19 patients.

Based on the above and due to the lack of information about vitamin D status in people residing in Homs City, this study was conducted. The aim of the study was to investigate the serological levels of 25(OH) D3 in a group of patients' companions who consulted the National Hospital of Homs.

The present work was approved by the Ethics Committee of Al Wataniya Private University in Syria (No. HERC4), with the date of issue being 15 May 2022.

2. MATERIALS AND METHOD:

A cross-sectional study was conducted in Homs, located in the middle region of Syria at latitude 34.7324° north, between June and October 2022. Patients' companions, ostensibly healthy individuals, were recruited at the National Hospital of Homs. Using a questionnaire distributed to those interested in participating, 690 subjects between the ages of 20 and 68 were included in this research (Figure 1). Exclusion criteria encompassed patients with chronic diseases such as diabetes, hepatitis, or chronic renal disease, and those taking medications that affect vitamin D metabolism or absorption, such as anticonvulsants, corticoids, oral contraceptives, or vitamin D supplements, including an extended-release form during the six months preceding data collection. Subjects with vitamin D intoxication, pregnant women, and individuals under 20 were not included in this study.

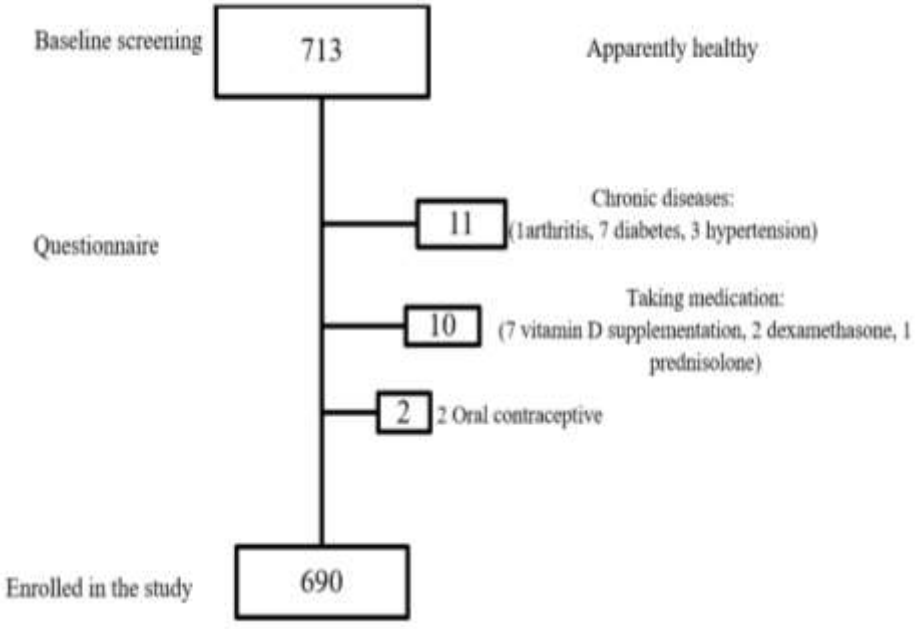

Figure 1. Subjects Recruitment

After obtaining personal written informed consent from all participants, 2-3 ml of venous blood samples were drawn using the Vacumed® blood collection system from FL MEDICAL, Italy. Subsequently, the samples were

centrifuged for 10 min at 1,700 ×g and stored at -20 °C until analysis. Serum 25(OH) D3 levels were analyzed using a chemiluminescent assay system (IMMULITE® 1000 immunoassay systems, Siemens, Germany), and 25(OH) D3 values were expressed as ng/ml.

Participants were divided into five groups according to age:

Group 1: Between 20 and 29 years old, Group 2: Between 30 and 39 years old, Group 3: Between 40 and 49 years old, Group 4: Between 50 and 59 years old, and Group 5: ≥ 60 years old. Additionally, women were classified into two groups: veiled (wearing a scarf on the head and covering the whole body) and non-veiled.

Vitamin D deficiency was classified as follows [12,13]:

Vitamin D insufficiency: serum levels of 25(OH) D3 ≤ 30 ng/ml

Vitamin D deficiency: serum levels of 25(OH) D3 ≤ 20 ng/ml

Severe vitamin D deficiency: serum levels of 25(OH) D3 ≤ 10 ng/ml

These levels were divided into four groups:

Group I: 30-100 ng/ml (sufficient)

Group II: 20-30 ng/ml (vitamin D insufficiency)

Group III: 10-20 ng/ml (vitamin D deficiency)

Group IV: Less than 10 ng/ml (severe vitamin D deficiency)

The prevalence of subjects in each gender group was determined.

2.1. Statistical Analysis

GraphPad Prism 5.0 was used for statistical analysis [14]. The data were presented as mean values ± standard deviation (SD). Student's t-test (for independent samples), Chi-square, and Mann-Whitney U test were employed to compare clinical and demographic parameters between the two groups, while the Kruskal-Wallis test was used for comparisons involving more than two groups. A p-value less than 0.05 was considered significant, based on a two-tailed test.

3. RESULTS

The study included 690 individuals aged between 20 and 68 years, comprising 210 males (30.4%) and 480 females (69.6%). The average age for males was 38.2±12 years, for females it was 36.5±15.5 years, and 75.5% of participants were under 50 years old.

3.1. Distribution of participants from both genders based on 25(OH) D3 levels:

Figure 2 revealed that 30.5% of participants from both genders exhibited the highest prevalence of vitamin D deficiency based on 25(OH) D3 levels.

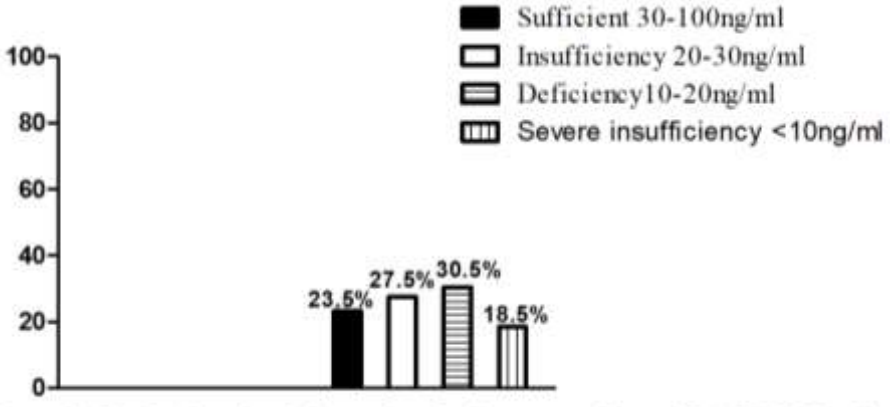

Figure 2: Distribution of participants from both sex according to 25 (OH)-D3 levels

3.2. Distribution of 25(OH)D3 levels according to age-group:

The lowest average level of vitamin D (11.79±8.79 ng/ml) was observed in the 20-29 years age group, while the highest average level was noted in the 40-49 years age group. The maximum prevalence (66.6%) of vitamin D deficiency was observed in the 50-59 years age group, whereas the lowest prevalence was seen in the 40-49 years age group (Figure 3). No significant differences in 25(OH) D3 levels among age groups were identified.

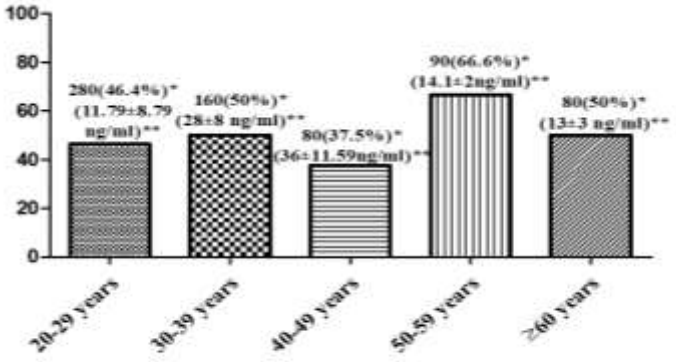

Figure 3. Distribution of vitamin D deficiency in each age group
***Number of participants(percentage of subjects with vitamin D deficiency)**
**** (average level of vitamin D ±standard deviation)**

3.3. Distribution of 25(OH)D3 levels according to gender:

The results demonstrated a significant statistical difference between females and males in terms of serum levels of 25(OH)D3, with values of 11.345±2.3 ng/ml for females and 39.65±11.28 ng/ml for males, respectively ($p < 0.0001$, Figure 4).

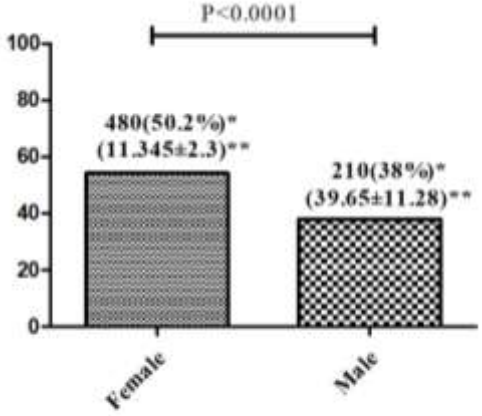

Figure 4. Distibution of vitamin D deficiency in each gender
***Number of participants(percentage of subjects with vitamin D deficiency)**
**** (average level of vitamin D ±standard deviation)**

3.4. Distribution of gender according to 25(OH)D3 levels:

Figure five revealed insufficient vitamin D levels in 18.8% of females and 8.7% of males. The prevalence of females suffering from vitamin D deficiency was approximately 19%, compared to 11.5% of males (the average level for both genders was 14.67±2.68 ng/ml). A significant severe vitamin D deficiency was reported in approximately 18.5% of females (mean level was 6.86±1.89 ng/ml), which was not detected in males who participated in this study.

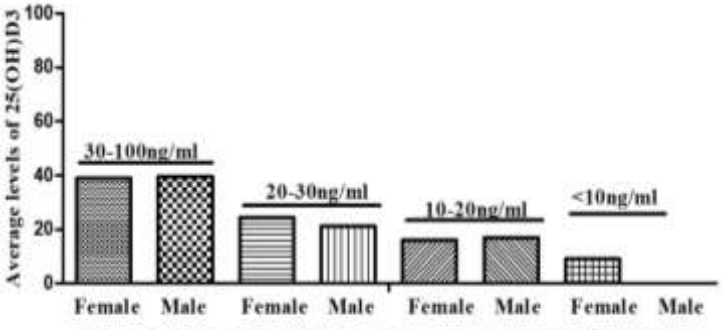

Figure 5. Distribution of both genders according to 25 (OH) D3 levels

3.5. Serum levels of vitamin D in veiled compared to non-veiled women:

In this study, 65% of females wore the hijab and exhibited serum levels of vitamin D lower than non-veiled females, with values of 11.3±2.5 ng/ml versus 25.5±3.2 ng/ml, respectively $p<0.0001$ (figure 6).

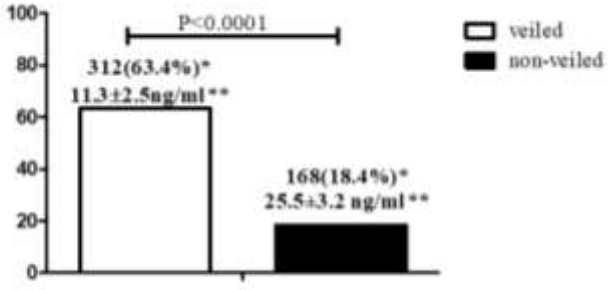

Figure 6: Distribution of vitamin D deficiency in veiled vs non-veiled women
***Number of participants(Percentage of subjects with vitamin D deficiency)**
**** (average level of vitamin D ±standard deviation)**

The female gender and clothing style were identified as independent risk factors for vitamin D deficiency, with p-values of 0.004 and <0.0001, respectively.

4. DISCUSSION

The primary finding of this research is the high prevalence of vitamin D deficiency identified in the study group, despite the predominantly sunny weather in Homs city throughout the year. The results indicated that 76.5%

of participants had an average vitamin D level of 22.4±6.4 ng/ml, signifying vitamin D inadequacy, with 30.5% exhibiting vitamin D deficiency (mean level 14.67±2.68 ng/ml). Severe vitamin D deficiency was observed in 18.5% of females in the study samples. Additionally, levels of 25(OH) D3 in females were significantly lower than those in males (11.345±2.3 ng/ml versus 39.65±11.28 ng/ml, respectively).

The findings of this study highlighted the lowest levels of vitamin D in the young population aged 20-29 years, with 46.4% of subjects having the lowest mean vitamin D level (11.79±8.79 ng/ml). Furthermore, vitamin D deficiency was observed in 54% of women and 50% of men under 35 years old. Additionally, 30.7% of females at reproductive age had levels ≤ 5 ng/ml. These low vitamin D levels could pose a risk of low bone density, fractures, and other complications associated with vitamin D deficiency.

This could be explained by common lifestyle patterns among the population. Nowadays, there is a notable inclination to avoid main meals containing ingredients that provide the body with vitamin D3, such as milk and eggs, or stimulate its absorption, such as tuna. This was corroborated by a study that identified a negative association between insufficient daily calcium and calorie intake with the concentration of vitamin D in older adults [15]. Additionally, there is a significant reliance on fast-food restaurants that are widely distributed in Syrian cities, including Homs, and offer reasonable prices. In fact, besides its negative impact on physical health, this food is rich in calories but poor in essential body nutrients and minerals, constituting a risk factor for vitamin D deficiency [16]. However, these aspects were not evaluated in this research.

Another crucial factor related to vitamin D is sun exposure, which significantly contributes to the endogenous production of the vitamin, thus fulfilling the daily requirements of vitamin D [17]. The impact of sunlight was evident in the presented work, as the levels of 25(OH)D3 in veiled women were lower than in non-veiled women, with values of 11.3±2.5 ng/ml versus 25.5±3.2 ng/ml, respectively. Moreover, 63.4% of women who wore the hijab suffered from deficiency. However, even non-veiled women exhibited vitamin D inadequacy, and 18.4% of them had vitamin D deficiency.

Apart from inadequate exposure to sunlight, dark skin, which is common in Mediterranean countries, and the use of sunscreen have also been proposed as reasons for low levels of vitamin D endogenously synthesized by the skin.

The results have also revealed the highest prevalence (66.6%) of vitamin D deficiency in the 50-59 years age group. This emphasizes the importance of vitamin D supplementation at this stage of life.

The obtained results were consistent with research conducted on a group of Syrians aged 18-62 from Damascus City. The outcomes showed that the majority of participants (61%) had vitamin D levels less than 10 ng/ml, 99.2% were below 30 ng/ml, and vitamin D deficiency was more prevalent in females than in males [18].

Comparing the results of this research with other studies carried out in neighboring countries, some findings are presented.

In Lebanon, several studies investigated the status of vitamin D levels in the Lebanese population at different ages and from both genders. A study on a group of samples aged between 19 and 60 years found an elevated percentage (83.5%) of vitamin D inadequacy and 63% of vitamin D deficiency in the studied population for both genders. Additionally, females between 19-39 years represented the highest prevalence (71.2%) of vitamin D deficiency [19]. Another research included a random sample of Lebanese adults, both females and males, with a mean age of 45.3 ± 15 years residing in the Greater Beirut area, and found that 39.1% of participants were deficient, using a conservative cut-off of 12 ng/ml [20]. In agreement with our results, low levels of vitamin D were highly represented in the Lebanese population at different ages.

In Jordan, recent research revealed an elevated

prevalence (89.7%) of low vitamin D levels (<30 ng/ml) among Jordanian adults. The highest distribution of vitamin D deficiency (<20 ng/ml) was found in females, which was selected as an independent risk factor associated with low vitamin D levels [21].

In Iran, several meta-analyses and systematic reviews involving more than 26,000 individuals reported that vitamin D deficiency was present in more than half of the study population, with the majority being female. Moreover, vitamin D deficiency was highly prevalent among the young and middle-aged (20-50 years), and the distribution of deficiency was significantly different between different geographical areas [22,23].

Concerning the Arabian Gulf region, a cross-sectional analysis including 102,342 participants attending primary healthcare centers in Qatar investigated vitamin D status in adults aged 18 - 65 years old. The study revealed that 14.1% suffered from severe vitamin D deficiency, 71.4% presented vitamin D deficiency, and 92.7% had vitamin D insufficiency. The higher prevalence rate of severe vitamin D deficiency (28.4%) was observed in young females between 19-28 years old [24]. In the United Arab Emirates (UAE), a large study conducted in Abu Dhabi found a high prevalence rate (72%) of participants being deficient and (10%) insufficient in vitamin D, with no difference between genders [25]. A new, interesting research aimed to evaluate the distribution of vitamin D deficiency and related risk factors among female migrants from Arab, South Asian, and Philippines countries inhabiting the UAE. Vitamin D deficiency was significantly prevalent in the study population, particularly in Arabs (87%) and South Asians (83%). Some associated risk factors identified were low physical exercise and being obese (BMI $\geq$ 30) [26].

In Kuwait, a descriptive study on Kuwaiti adults assessed the prevalence of vitamin D deficiency and its associated socio-demographic and daily lifestyle risk factors by measuring serum levels of 25-hydroxyvitamin D (25(OH) D3). The outcomes showed that vitamin D deficiency was highly distributed among adults, with some risk factors involving the age of 23-39 years, being single, consuming fast food, clothing style, and an indoor working environment [16]. A cross-sectional analysis studied the related risk factor of vitamin D deficiency in the Kuwaiti population aged over 65. Findings revealed that 63% of participants had vitamin D deficiency, and those who had not received vitamin D supplementation presented the highest prevalence [27].

In Saudi Arabia, one of the largest cross-sectional studies included participants of all ages and followed their vitamin D levels from 2008 to 2017. The results provided hope, as some improvement in the prevalence of vitamin D deficiency across all ages and both genders had been found. However, low levels of 25(OH)D3 remain a considerable public health problem in Saudi Arabia. Moreover, young teenagers under 18 years old presented a higher prevalence of vitamin D deficiency than the elderly [28].

Regarding the Arab countries of North Africa, the situation is not better. A study in Morocco investigated the relationship between sun exposure and vitamin D status among 331 Moroccan adults, finding that hypovitaminosis D was very prevalent, representing 94% of the study population, especially in females. Clothing code is attributed to vitamin D deficiency, as 76.4% of subjects exposed only their faces [29].

In Tunisia, a study involving 209 healthy participants found that 92.3% had 25(OH)D3 serum levels less than 30 ng/ml, and 47.6% had levels <10 ng/ml. The distribution of deficiency was statistically higher in females than in males. The primary associated risk factors were veiling, inhabiting rural areas, and regular sunscreen application [30].

Hence, the results of studies carried out in neighboring countries are similar, and vitamin D deficiency is highly prevalent in the Middle-East region. According to several studies, the required serum levels of 25(OH)D3 must be at least 30 ng/ml to maintain a healthy body and normal bone

density, preventing fractures, muscle weakness, colon cancer, and dental health [31].

The strength of this study lies in the fact that the samples were collected during the same period when exposure to sunlight was substantial. Additionally, the participants in this research were apparently healthy; they did not have any chronic diseases that could prevent them from going out and being exposed to sunlight, nor had they received any medication that might affect vitamin D levels. They lived in different regions of Homs and were from both genders. Moreover, the 25(OH)D3 level was measured by the chemiluminescent method using the Immulite®/Immulite 1000 Systems, which is a gold standard laboratory method for measuring vitamin D.

5. CONCLUSION

This study draws attention to the significant prevalence of vitamin D inadequacy and deficiency among participants. Vitamin D deficiency was highly prevalent in the study group, despite the predominantly sunny weather in Homs city throughout the year. Moreover, females exhibited lower levels than males, and veiled women had lower serum concentrations than non-veiled individuals. Female gender and clothing patterns were identified as independent predictors of vitamin D deficiency. It is recommended to conduct a larger-scale study covering multiple Syrian governorates to assess the prevalence of vitamin D deficiency in Syria and examine the relationship between serum levels of vitamin D and socio-demographic and daily lifestyle factors. Measures are proposed, including food fortification policies for dairy products commonly consumed among Syrian citizens with vitamin D, and increasing community awareness of the crucial role of vitamin D supplementation after a certain age.

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No potential conflict of interest relevant to this article was reported.

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توزع مستويات فيتامين د لدى مجموعة من المجتمع السوري

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ملخص

هدف البحث: دراسة المستويات المصلية لـ 25–هيدروكسي فيتامين د3 لدى عينة من السوريين الأصحاء في مدينة حمص. وذلك عن طريق دراسة مقطعية لـ 690 مشاركًا – أصحاء ظاهرياً – حضروا إلى مستشفى حمص الوطني. تم قياس المستويات المصلية لـ 25–هيدروكسي فيتامين د3 باستخدام المقايسة المناعية الكيميائية. بينت النتائج أنّ معدل الانتشار العام لنقص فيتامين (د) (القصور والنقص والنقص الشديد) في العينة المدروسة بلغ 76.5٪. علاوة على ذلك، فإن 49٪ من العينات كانت تعاني من نقص فيتامين (د)، 18.5٪ منها كانت تعاني من نقص حاد. بالإضافة إلى أن مستويات 25–هيدروكسي فيتامين د3 للإناث أقل من الذكور (11.3 ± 2.3 نانوغرام / مل مقابل 39.6 ± 11.28 نانوغرام / مل، على التوالي، .($p<0.0001$) وقد كانت المستويات المصلية لدى النساء المحجبات أقل من غير المحجبات، 11.3 ± 2.5 نانوغرام / مل مقابل 25.5 ± 3.2 نانوغرام / مل، على التوالي، .($p<0.0001$) كما تبين أن جنس الأنثى ونمط اللباس من عوامل الخطر المستقلة لنقص فيتامين (د). إنّ نقص فيتامين (د) كان شائعًا جدًا في العينة المدروسة، على الرغم من الطقس المشمس في مدينة حمص معظم العام إلا أنّه يجب متابعة البحث ليشمل مجموعة أكبرمن المحافظات السورية لدراسة عوامل الخطر المتعلقة بنمط الحياة والسلوكيات الاجتماعية والثقافية المرتبطة بنقص فيتامين د.

الكلمات الدالة: 25–هيدروكسي فيتامين د 3، نقص فيتامين د، عوز فيتامين د، أشعة الشمس، سوريا.

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Cytotoxicity, Antioxidant Activities, GC-MS and HPLC Fingerprint Analyses of Different Extracts of *Desmodium tortuosum* (Sw.) DC

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ABSTRACT

The family Fabaceae is the third-largest flowering plant family, and the genus Desmodium has exhibited a wide range of biological activities and a variety of chemical constituents. In the present study, different extracts of Desmodium tortuosum were evaluated for their cytotoxic and antioxidant activities, as well as their total phenolic content (TPC). The antioxidant activities were estimated using the 1,1'-diphenyl-2-picraylhydrazyl free radical (DPPH), while the cytotoxic activity was evaluated via the brine shrimp lethality test (BSLT). The antioxidant activity results revealed that the DPPH radical scavenging activity (SC50) ranged from 1.12 to 61.22 µg/ml with respect to ascorbic acid (SC50 = 7.45 µg/ml). Among all tested fractions, 90% methanol was the most active. On the other hand, the cytotoxic activities were arranged as follows: n-BuOH (LC50 = 310), EtOAc (LC50 = 350), and 70% methanol (LC50 = 380). High-Performance Liquid Chromatography-Fingerprint analyses were used to determine the chemical composition and relative proportions of phenolic compounds. GC-MS analysis indicated the presence of fatty acids and other compounds. The major identified compounds were Benzene (1-butyloctyl) (11.88%) and Himachalene <α-> (11.08%) for the ethyl acetate extract and 10-Undecenoic acid, methyl ester (25.50%) for unsaponifiable matter.

Keywords: Antioxidant, Cytotoxicity, *Desmodium tortuosum*, GC-MS; HPLC.

INTRODUCTION

The family Fabaceae is the third-largest flowering plant family, consisting of about 482 genera and 1,200 species of evergreen trees, herbs, water plants, and shrubs [1]. A wide array of nutrients, such as proteins, amino acids, and fatty acids, are found in Fabaceae plants [2]. Among them, fatty acids fundamentally compose lipid molecules, hormones, and cell membranes, serving as an energy source for cells and playing a key role in energetic metabolic and structural activities [3]. Many species in this family are renowned in traditional medicine for their use as anti-perspirants, diuretics [4], and in the treatment of nephritis [5], diabetes, leukemia, uterine cancer [6], diarrhea, cough, cramps, and sores of the mouth [7]. Fabaceae plants are found all over the world, growing in various environments and climates [8]. Legume products contribute to the world economy through food, pharmaceuticals, medicine, chemicals, and fertilizers. Legumes are also utilized as insecticides, molluscicides, and anti-fungal agents [1].

The genus Desmodium contains about 350 species, mainly distributed in tropical and subtropical regions worldwide, with approximately 28 species in China. Most of its plants are herbs, shrubs, or sub-shrubs, but rarely trees. Besides their popularity as feeding stuffs, they are also used in traditional medicine [9]. Desmodium styracifolium (Osbeck) Merr. has been recorded in the Chinese Pharmacopeia for the treatment of urolithiasis, painful urination, and cardio-cerebrovascular diseases

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[10]. Chemical studies of D. styracifolium have shown the presence of isoflavanones, coumaronochromone [11], saponin, and alkaloids [12-13]. Other species of Desmodium, including D. gangeticum and D. adscendens, are used ethnomedicinally worldwide. Phytochemical research on both species has led to the isolation of alkaloids, phospholipids, sterols, flavones, and triterpenoid saponins. They exhibit a wide spectrum of in vitro and in vivo pharmacological activities, such as antileishmanial, immunomodulatory, smooth muscle relaxant, anti-inflammatory, anti-ulcer, antidiabetic, antiviral, antioxidant, and hepatoprotective activities [14].

During the past 30 years, the Brine Shrimp Lethality Assay has been widely used as a toxicity test for a variety of plant products. This test has been successively employed for bioassay-guided fractionation of active cytotoxic and antitumor agents, demonstrating several advantages such as rapidness, simplicity, low requirements, robustness, where the cysts are commercially and readily available, inexpensive, and with high degrees of repeatability [15].

The overproduction of reactive oxygen species (ROS), such as superoxide anion (O2), hydrogen peroxide, hydroxyl radical (OH), and peroxyl radical (ROO), may induce oxidative stress in the human body, consequently causing degenerative and pathological damages, such as aging, cancer, cardiovascular diseases, Alzheimer's disease, and inflammation. Certain environmental factors, such as stress, cigarette smoking, and some drugs, are also associated with the elevation of free radicals in the human body, so antioxidants play an important role in protecting the body from oxidative stress. Recently, antioxidant activities of medicinal plants or plant-derived chemical compounds and health foods are being investigated comprehensively [16].

The characterization of oils and fats has mainly focused on the principal components, which constitute the saponifiable fraction comprising over 95% of oils and fats. The unsaponifiable matters present in vegetable oils and fats are usually composed of sterols, fatty alcohols, tocopherols, triterpene alcohols, and hydrocarbons, each with individual biological importance [17-18]. Desmodium tortuosum plants were recently cultivated in Egypt, and there is limited information in the literature regarding their chemical content and biological activities. The present study aims to determine the chemical content and evaluate the total phenolic contents, cytotoxic and antioxidant activities of their different extracts. Conventional gas chromatography–mass spectroscopy (GC-MS) was adopted for composition analysis to identify the content of aliphatic and fatty acid compounds, while High-Performance Liquid Chromatography-Fingerprint Analyses was used to quantify different groups of phenolic components in the plant.

MATERIALS AND METHODS

Chemical, reagents and equipments

All solvents and reagents used were of analytical grade. 1,1'-diphenyl-2-picrylhydrazyl (DPPH) free radical and Folin-Ciocalteu's reagent (FCR) were purchased from Sigma-Aldrich Co. Gallic acid and ascorbic acid were purchased from Merck Chemical Co. All solvents and acids (methanol, petroleum ether, chloroform, ethyl acetate, n-butanol) were obtained from Sigma-Aldrich Co. The absorbance measurements for antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601 (Milton Roy, USA).

Plants materials

Desmodium tortuosum (Sw.) DC peels were collected from Elephantine Island in Aswan, Egypt. The plant was added to the Egyptian flora as a new species in Egypt in 2004. The identity of the plant was established by Dr. Fatma Abdallah Mohamed, Lecturer in the Plant Department, Faculty of Science, Aswan University. A voucher specimen was kept in the Department of Medicinal Chemistry, Theodor Bilharz Research Institute (TBRI) under the number (Dt-2012). The plant materials were air-dried in a shaded place at room temperature and then powdered using an electric mill. Finally, they were stored in a tightly closed container in a dark place until the extraction process.

Extraction

Small-scale extraction was carried out by taking samples from the dry powder of the fresh leaves of the plant (10 g). The samples were then separately extracted with different solvents (100 ml x 4): 100% methanol and methanol-water mixtures (90%, 85%, and 70%) at room temperature for one week with daily shaking. The extracts were filtered, and the extraction process was repeated four times. Each extract was filtered using Whatman filter paper No.1 and concentrated using a rotary evaporator (Buchi, Switzerland) at (50 ± 2°C), resulting in a known weight of each crude methanol extract. The crude extracts were collected and stored at room temperature in the dark for further processing.

Air-dried plant leaves (0.25 kg) were extracted by soaking in aqueous methanol (MeOH: water; 90: 10; v/v) at room temperature. After filtration, the collected filtrate was concentrated using a rotary vacuum evaporator under pressure and low temperature. The dried 90% methanol extract (31 g) underwent a defatting process to remove unwanted substances using petroleum ether (60-80°C). Subsequently, the fractionation process was performed by dissolving 28 g of the 90% defatted extract in distilled water. The completely soluble part was then partitioned using dichloromethane, ethyl acetate, and n-butanol, resulting in 2.81 g, 2.0 g, 3 g, and 10 g for dichloromethane, ethyl acetate, and n-butanol, respectively.

The n-butanol extract (7 g) was fractionated on a polyamide column chromatography [(Ø 7.0 × 120 cm)]. Elution started with water, followed by a gradual increase of methanol. Based on comp-TLC and PC with the use of UV light, individual 120 fractions (250 ml each) were collected into seven collective fractions (I-VII). Two groups of fractions (4-12 [I]) and (73-78[III]) (0.9 g & 1.3 g, respectively) were chosen for further chemical and biological analysis with other extracts.

Extraction of lipid constituents

About 50 g of the dried, powdered Desmodium tortuosum were extracted with n-hexane in a Soxhlet apparatus. The combined n-hexane extract was passed through fuller's earth to remove the colored pigments, filtered, dried over anhydrous sodium sulfate, and evaporated under vacuum at 40°C until dryness to create a pale-yellow residue (35 g).

Saponification of *n*-hexane extract

The n-hexane extract (25 g) was saponified by refluxing with 100 ml N/2 alcoholic KOH. The alcoholic solution was concentrated to about 20 ml and diluted with cold distilled water. The unsaponifiable constituents were extracted by partition with successive portions of diethyl ether (3×100 ml). The combined ether extract was washed with distilled water, dehydrated over anhydrous sodium sulfate, and evaporated under vacuum until dryness to give a yellowish-brown semi-solid residue of unsaponifiable matter (5 g), which was subjected to GC analysis.

Assessment of the total phenolic contents

The total phenolic content of the extracts was quantified using Folin-Ciocalteu's method adapted to a 96 well–plate. This method closely follows that used by previous workers, Diko et al. 2002 [19]. Thus, 20 µL of each plant extract dissolved appropriately in distilled water was mixed with 100 µL F-C reagent freshly diluted 1/10 with distilled water. After 5 min incubation at room temperature, 80 µL of 7.5% Na2CO3 solution was added. The whole was left for 30 min at room temperature in the dark with slight shaking. The absorbance was measured at 735 nm in a microplate-reader (Biochrom Asys UVM 340) against blank. Gallic acid was used as the standard. The results (average of triplicate analyses) were expressed in mg/g of extract, gallic acid equivalent (GAE).

Antioxidant activity

Free radical scavenging antioxidant activity using DPPH assay

The scavenging activity of the stable 1,1'-diphenyl-2-picrylhydrazyl free radical was determined by the method described by Marwah et al. 2007 [20]. Briefly, the reaction medium contained 2 ml of 100 µM DPPH purple solution in methanol and 2 ml of plant extract; ascorbic acid was used as

the standard. The reaction mixture was incubated in the dark for 20 min, and the absorbance was recorded at 517 nm. The assay was carried out in triplicate. The DPPH radical scavenging activity was calculated according to the equation: % DPPH radical scavenging activity = [1 - ($A_{sample}/A_{control}$) x 100], where $A_{control}$ and A_{sample} are the absorbencies of control and sample after 20 min, respectively. The SC50 (concentration of the sample required to scavenge 50% of DPPH radicals) values were determined. The decrease in the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity.

Cytotoxicity (Brine shrimp lethality test)

Brine shrimp lethality bioassay test A solution of instant ocean sea salt (Aquarium System, Ohio) was made by dissolving 2.86 gm in distilled water (75 ml). Fifty milligrams of Artemia salina Leach eggs (Artemia, Inc., California) were added to a hatching chamber. The hatching chamber was kept under an inflorescent bulb for 48 hrs for the eggs to hatch into shrimp larvae. Twenty milligrams of the tested extract was dissolved in 2 ml of methanol or the solvent in which it was soluble, and from this, 500, 400, 300, 200, 100, 50, 5 µl of each solution was transferred into vials corresponding to 1000, 800, 600, 400, 200, 100, and 10 µg/ml, respectively. Each dose was tested in triplicate. The vials and the control containing 500 µl of the solvent were allowed to evaporate to dryness in about 48 hrs at room temperature. Four and a half milliliters of instant ocean sea solution were added to each vial, and 10 larvae of Artemia salina (taken 8-72 hrs after the initiation of hatching) were added to each vial. The final volume of solution in each vial was adjusted to 5 ml with sea salt solution immediately after adding the shrimp. Twenty-four hours later, the number of surviving shrimp at each dosage was counted and recorded. LC50 values were determined with 95% confidence intervals by analyzing the data. The data were analyzed, and LC50 values were calculated and carried out according to the Reed-Muench method. Potassium dichromate was used as the standard [22-23].

HPLC-DAD-ESI/MS/MS conditions

Separation and determination of phenolic compounds were performed by reverse-phase HPLC (RP-HPLC)/diode array detection (DAD) (Hewlett Packard 1050) using an Alltima C18, 5mm column (150 mm × 4.6 mm id) with a guard column Alltima C18, 5mm (Alltech). The solvent system used was a gradient of A (CH3 COOH 2.5% v/v), B (CH3 COOH 8% v/v), and C (acetonitrile). The best separation was obtained with the following gradient: at 0 min, 5% B; at 20 min, 10% B; at 50 min, 30% B; at 55 min, 50% B; at 60 min, 100% B; at 100 min, 50% B and 50% C; at 110 min, 100% C until 120 min. The solvent flow rate was 1 ml/min, and the separation was performed at 35°C. The injection volume was 10 µl for each sample solution. Phenolic compounds were assayed by external standard calibration at 280 nm and expressed in µg/100ml. All values were the mean of two injections [23]. HPLC analysis was carried out according to the reported procedures [24-26].

GC-MS analysis

Gas-chromatography-mass spectroscopy (GC-MS) analysis was performed according to the reported procedures [27-28]. On the other hand, fatty acid compositions of the three oils were investigated via GC-MS analysis according to the method described [29&17]. The identification of the chemical components and the interpretation of GC-MS spectrum were carried out according to the database of National Institute Standard and Technique (NIST08s) (WILEY8) [30].

Statistical analysis

The obtained antioxidant and total phenolic contents results were presented as mean ± S.D., and the statistical procedures were performed using SPSS 13.0 program. Computations were based on Finney, 1971 [31]. For comparisons, the Chi2 test (pairwise versus control) was performed. Data were presented as a percentage. The limit for statistical significance was set at $p \leq 0.05$ (significance level of 95%).

RESULTS AND DISCUSSION

1. Quantification of total phenolic contents (TPCs)

The results in Table (1) showed the total phenolic contents date as the following order, [4 -12] fractions (162.63 ± 2.38)

> [73 -78] fractions (115.70 ±7.15) > 85 % methanol (97.79 ± 6.31) > 90% MeOH (88.15 ± 2.38) > EtOAc (64.74 ± 6.31) > Pet. ether (61.98 ± 4.13) > 70 % methanol (60.15 ± 1.88) > *n*-BuOH (57.75 ± 3.98) (mg GAE /g dry extract).

2. Antioxidant activity

2.1. Free radical scavenging antioxidant activity using DPPH assay

The results in Table (1) revealed that the antioxidant activities (expressed as SC_{50} values in µg/ml) were arranged in the following order; 90% MeOH (1.12 ± 2.36 µg/ml) > CH_2Cl_2 (2.12 ± 4.08 µg/ml) > Pet. ether (2.53 ± 4.08 µg/ml) > *n*-BuOH (2.93 ± 4.09 µg/ml) > EtOAc & Fraction [73-78] (3.46 ± 4.08 µg/ml) > Fraction [4-12] comparison with ascorbic acid as standard with SC_{50}= 7.50 µg/ml.

Table (1): *In vitro* antioxidant activities and total phenolic contents of different extracts and fractions of *D. tortuosum*

Sample	DPPH SC_{50} [µg/ml][a]	TPC (mg GAE /g dry ext.)[b]
100% MeOH	30.90 ± 2.81	1.31 ± 0.18
90% MeOH	1.12 ± 2.36	88.15 ± 2.38
85% MeOH	61.22 ± 4.08	97.79 ± 6.31
70% MeOH	40.81 ± 4.08	60.15 ± 1.88
Pet. ether	2.53 ± 4.08	61.98 ± 4.13
CH_2Cl_2	2.12 ± 4.08	39.94 ± 2.38
EtOAc	3.46 ± 4.08	64.74 ± 6.31
n-BuOH	2.93 ± 4.09	57.75 ± 3.98
Fr. III	3.46 ± 4.08	115.70 ± 7.15
Fr. I	3.76 ± 6.22	162.53 ± 2.38
Ascorbic acid	7.50	-------

Results are presented as mean ± S.D. (n = 3).
[a]DPPH results values are presented in SC_{50} values (µg/ml).
[b]TPC results are presented as mg gallic acid equivalent/g dry extract (mg GAE/g ext.).

Cytotoxicity

1. Brine shrimp lethality bioassay test

The results present in Table (2) revealed that the cytotoxic activities of the tested samples are in the order: *n*-BuOH (LC_{50}= 310), EtOAc (LC_{50}= 350), 70 % methanol (LC_{50}= 380). Both 85 % and pure methanol extracts showed very week activity.

Table (2): Mortality percent of Brine Shrimp of different extracts of *Desmodium tortuosum* plant

Dose ext. Conc.	Mortality								
	100%MeOH	70%MeOH	85%MeOH	Pet. ether	CH_2Cl_2	EtOAc	*n*-butanol	90%MeOH	H_2O
1000	68.12	98.81	65.12	79.17	63.79	100	100	100	12.12
800	26.85	98.18	29.41	40.90	41.18	97.5	98.81	100	5.08
600	22.22	71.70	6.06	7.14	22.08	92.72	96.43	61.70	0
400	6.38	44.78	0	0	7.77	58.97	65.79	33.33	0
200	0	30.93	0	0	0	9.1	18.18	4.69	0
100	0	23.62	0	0	0	0	0	0	0
20	0	19.11	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
LC_{50}	850	380	880	820	825	350	310	500	-

HPLC-fingerprint analysis

In this study, fourteen standard phenolic compounds were used as reference compounds (Table 3 and Figure 1). The obtained results revealed variable amounts of these standard compounds present in the tested samples (Table 3 and Figures 2-4).

Table (3): Areas under peaks and concentrations of the ethyl acetate extract, fractions [III] and fractions [I] of *Desmodium tortuosum* against fourteen standard phenolic compounds

Compounds	Standard			EtOAc		Fr. III		Fr. I	
	Rt,	Area	Conc.	Area	Conc .	Area	Conc.	Area	Conc.
Gallic acid	3.116	829.87	40	251.48	12.12	184.41	10.18	1117.40	61.67
Chlorogenic acid	3.515	335.32	46.9	000	0.00	38.63	6.18	3360.69	537.32
Catechin	3.844	612.25	148.5	49.66	12.05	000	0.00	000	0.00
Caffeic acid	4.973	1068.72	30	424.88	11.93	80.72	2.51	824.82	25.67
Syringic acid	5.357	882.99	30	725.16	24.64	98.10	3.78	1244.33	47.96
Rutin	5.774	983.74	120	136.13	16.61	000	0.00	631.64	86.11
Ellagic acid	6.901	757.02	70	59.08	5.46	42.89	4.45	000	0.00
Coumaric acid	7.680	946.65	20	120.48	2.55	21.57	0.53	526.83	12.86
Vanillin	8.387	1007.61	30	253.4	7.54	76.72	2.59	526.02	17.77
Ferulic acid	9.022	857.80	20	000	0.00	000	0.00	333.67	9.08
Naringenin	9.450	836.92	20	339.42	8.11	000	0.00	5373.64	150.72
Propyl gallate	10.277	409.00	8.33	599.80	12.22	1214.67	27.79	2154.92	49.31
Quercetin	10.711	868.80	60	2297.64	158.68	1304.04	100.53	7089.20	546.52
Cinnamic acid	11.200	1280.68	10	2044.55	15.96	2439.54	21.46	541.86	4.77

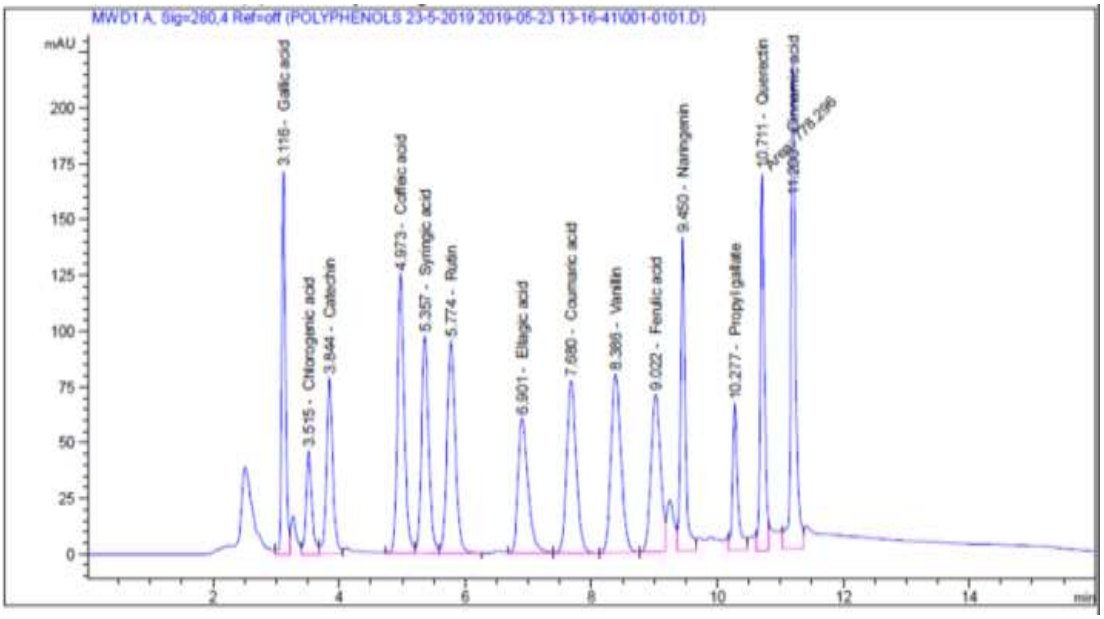


Fig. 1. High performance liquid chromatography chromatogram of fourteen standard phenolic compounds

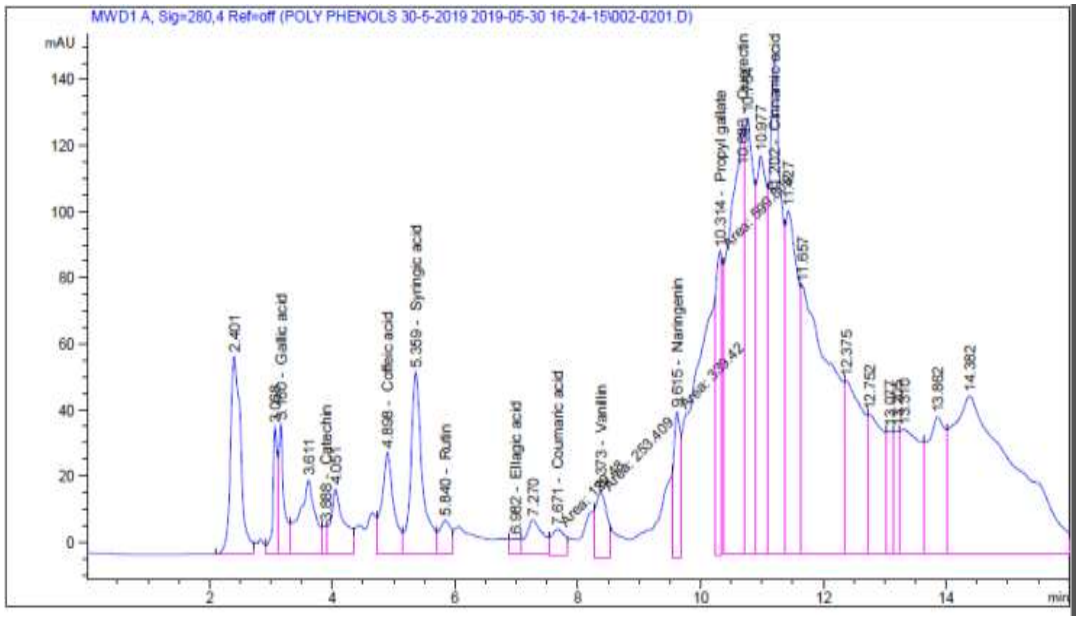


Fig. 2. High-performance liquid chromatography-fingerprint chromatogram of the ethyl acetate extract

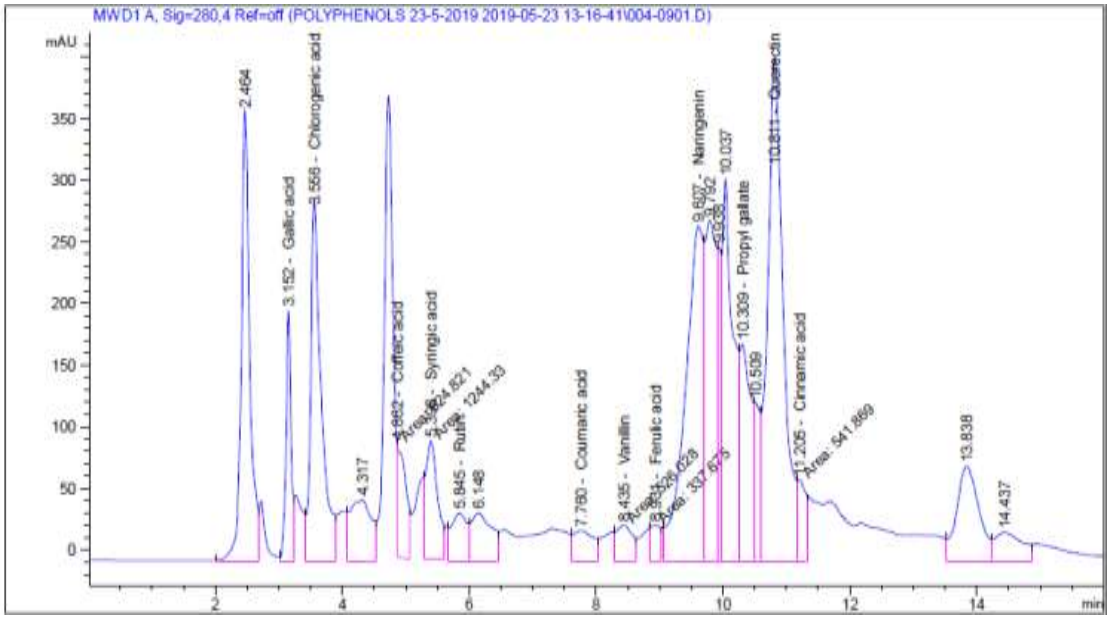


Fig. 3. High-performance liquid chromatography-fingerprint chromatogram of the *n*-butanol fractions (4-12)

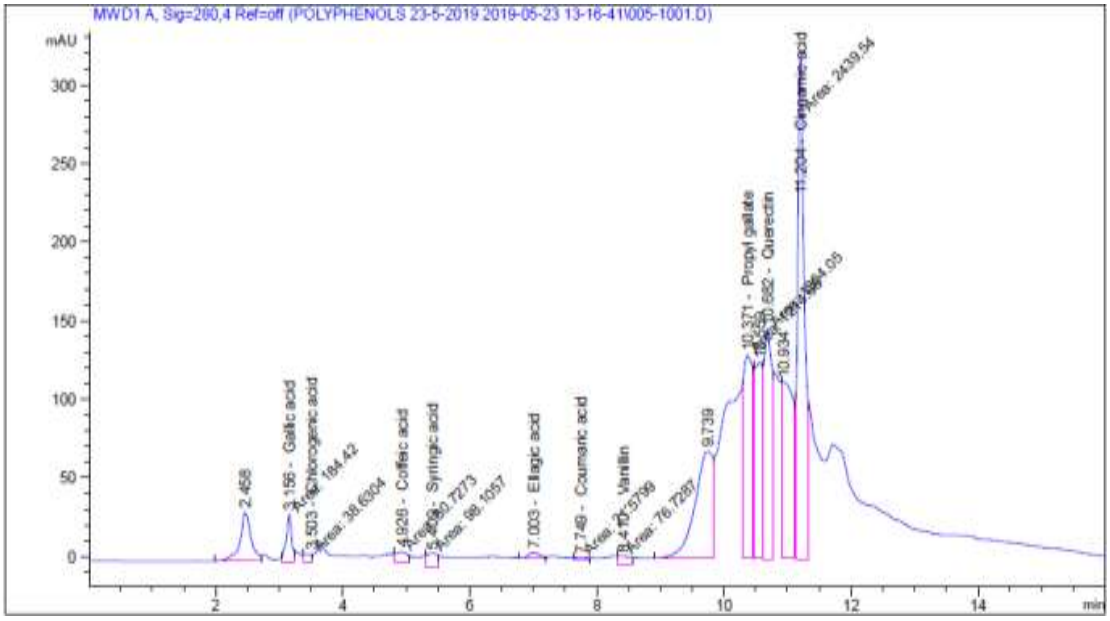


Fig. 4. High-performance liquid chromatography-fingerprint chromatogram of the *n*-butanol fractions (73-78)

5. GC-MS analysis

Chemical composition of the ethyl acetate extract of Desmodium tortuosum, as shown in (Figure 5 and Table 1S), indicates the presence of thirty-three compounds representing 89.44% of the total extract composition. The qualitative GC-MS analyses of the unsaponifiable extract resulted in the identification of thirty-two compounds (Figure 6 and Table 2S).

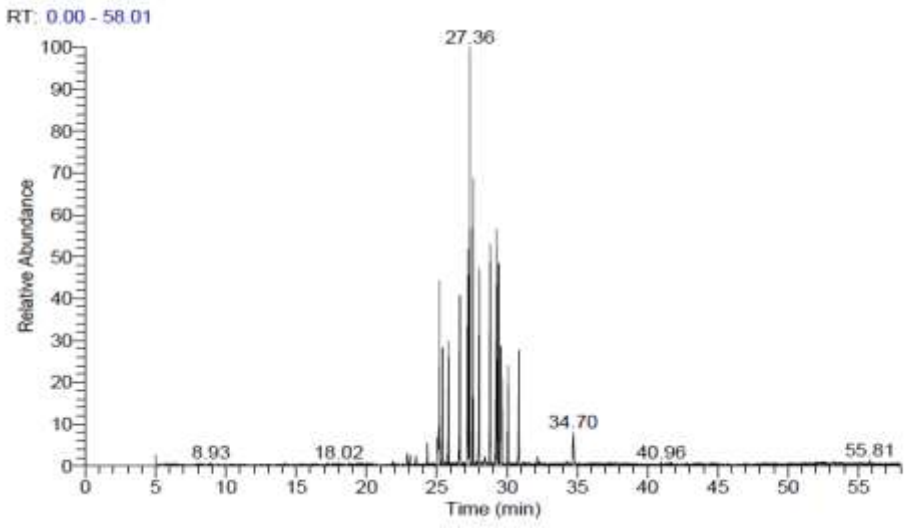
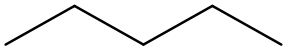
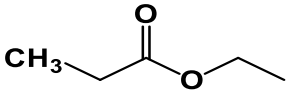
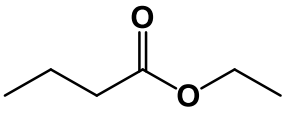
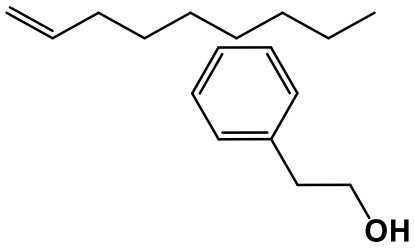
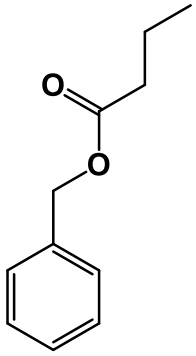
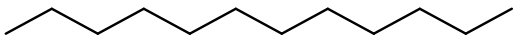
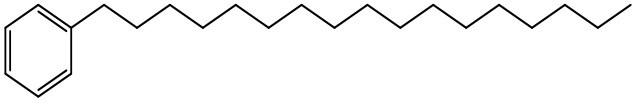
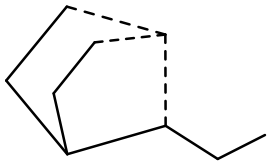
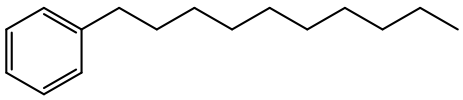
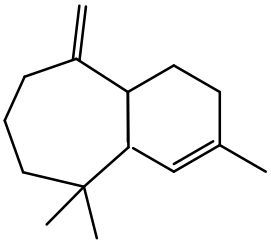
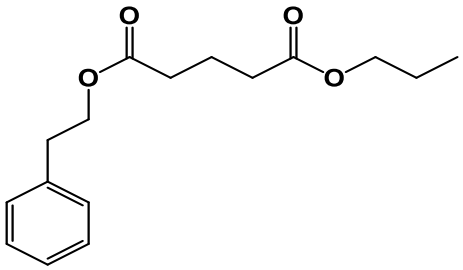
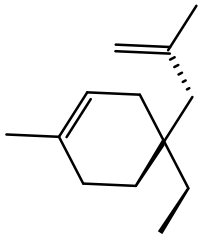
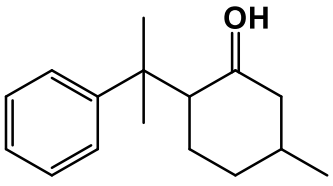
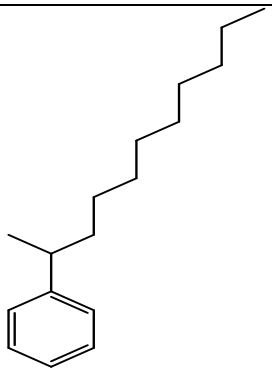
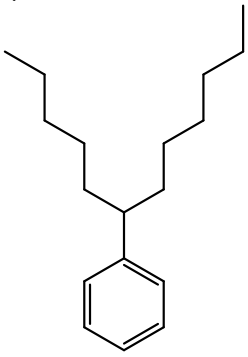
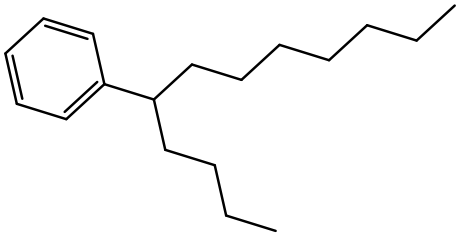
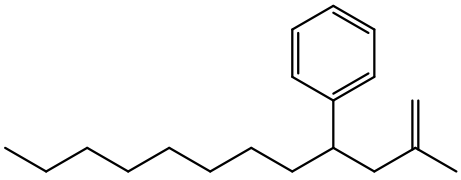
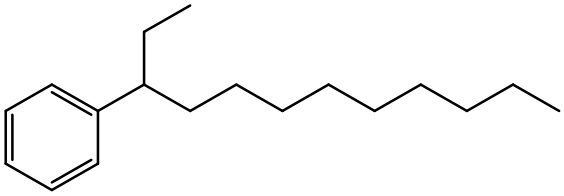


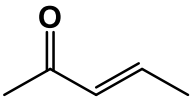
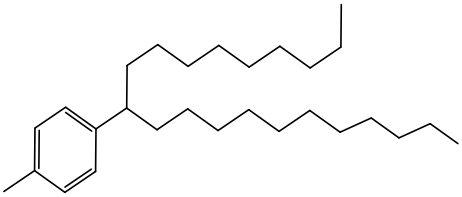
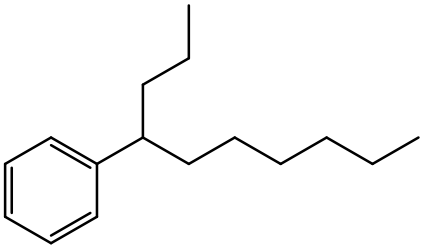
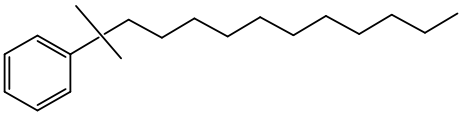
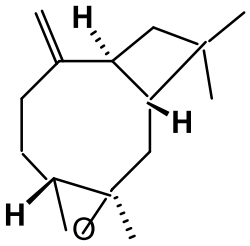
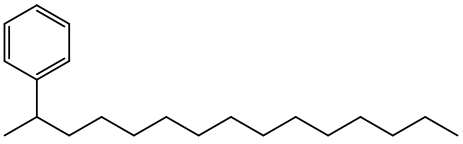
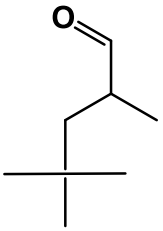
Fig. 5. GC-MS analysis of the ethyl acetate extract of *Desmodium tortuosum*.

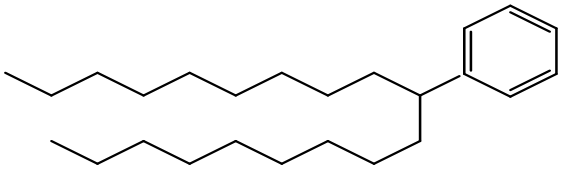
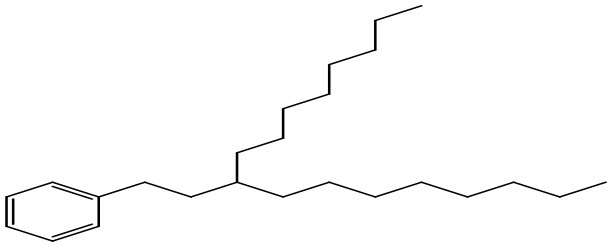
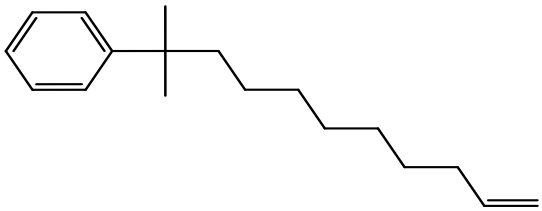
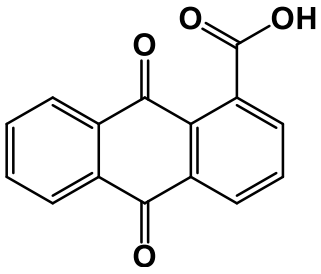
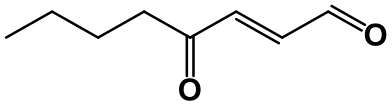
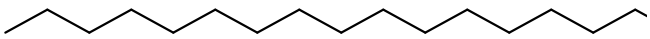
Table 1S: Chemical constituents identified in the ethyl acetate extract of *Desmodium tortuosum* using GC-MS analysis

No	M.W.	M.F.	R.T.	Area %	Name	Structure
1	86	C_6H_{14}	1.62	0.16	Hexane <N->	
2	102	$C_5H_{10}O_2$	2.1	0.07	Propionate	
3	130	$C_7H_{14}O_2$	4.38	0.08	Propyl Butrate	
4	128	$C_{8H6}O$	4.93	3.36	Heptanone<2-Methyl -4->	----
5	140	$C_{10}H_{2O}$	6.8	0.53	Decene	
6	122	$C_8H_{10}O$	10.98	7.43	Phenyl Ethyl Alcohol	
7	178	$C_{11}H_{14}O_2$	20.85	5.31	Benzyl Butyrate	
8	196	$C_{14}H_{28}$	22.95	0.21	Tetra-decene	
9	358	$C_{26}H_{46}$	23.01	0.33	Benzene,(1-propylheptadecyl)	

No	M.W.	M.F.	R.T.	Area %	Name	Structure
10	120	C_8H_8O	23.51	0.35	Norbornadiene7carbaldehyde	
11	218	$C_{16}H_{26}$	24.34	0.64	Benzene, decyl	
12	204	$C_{15}H_{24}$	25.2	11.08	Himachalene <α->	
13	320	$C_{19}H_{28}O_4$	25.58	0.13	Glutaric acid, isohexyl phenethyl ester	
14	204	$C_{15}H_{24}$	25.85	1.20	Acoradiene	
15	232	$C_{16}H_{24}O$	25.95	0.06	Cyclohexanol,5-methyl-2-(1-methyl-1-phenylethyl)	

No	M.W.	M.F.	R.T.	Area %	Name	Structure
16	232	$C_{17}H_{28}$	26.64	5.78	Benzene,(1-methyldecyl)	
17	246	$C_{18}H_{30}$	27.23	9.96	Benzene, (1-pentylheptyl)	
18	246	$C_{18}H_{30}$	27.35	11.88	Benzene, (1-butyloctyl)	
19	246	$C_{17}H_{26}O$	27.58	8.21	Undecanal, 3-phenyl	
20	246	$C_{18}H_{30}$	28.02	6.50	Benzene, (1-ethyldecyl)	

No	M.W.	M.F.	R.T.	Area %	Name	Structure
21	112	$C_7H_{12}O$	28.42	0.43	3-Hexen-2-one,5-methyl	
22	358	$C_{26}H_{46}$	28.89	0.07	Benzene,1-(1-heptyldodecyl) 4-methyl	
23	218	$C_{16}H_{26}$	29.64	2.28	Benzene, (1-propylheptyl)	
24	274	$C_{20}H_{34}$	30.08	3.05	Tridecane,2-methyl-2-phenyl	
25	220	$C_{15}H_{24}O$	30.62	3.73	Caryophyllene Oxide	
26	260	$C_{19}H_{32}$	30.84	3.42	Benzene, (1-methyldodecyl)	
27	128	$C_8H_{16}O$	31.17	0.07	2,4,4-Trimethyl-2-penten-1-ol	

No	M.W.	M.F.	R.T.	Area %	Name	Structure
28	348	$C_{23}H_{40}O_2$	31.28	0.09	Benzaldehyde dioctyl Acetal	
29	344	$C_{25}H_{44}$	31.53	0.16	Benzene, (3-octylundecyl)	
30	246	$C_{18}H_{30}$	32.01	0.09	Benzene,(1,1-dimethyldecyl)	
31	252	$C_{15}H_8O_4$	32.18	0.32	Anthraquinone-1-carboxylic acid	
32	140	$C_8H_{12}O_2$	34.71	2.30	4-Oxo-trans-2-octenal	
33	252	$C_{18}H_{36}$	38.6	0.07	Octadecene <1->	

9 Isoprene compounds
3 Monoterpene compounds
16 Sesquterpenes
2 Diterpene compounds
4 Sestraterpene compounds
15 Oxygenated compounds
18 Deoxygenated compounds
Total 89.44%

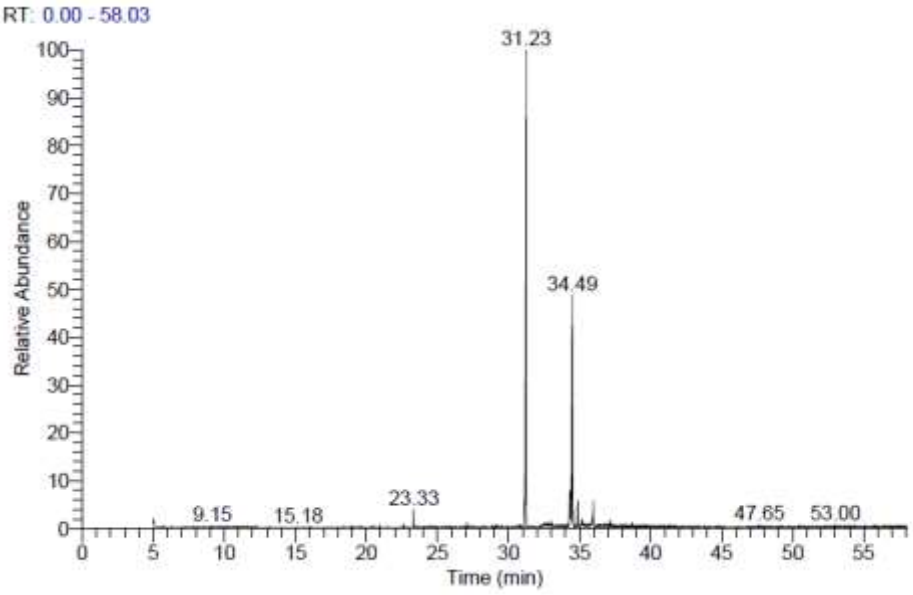
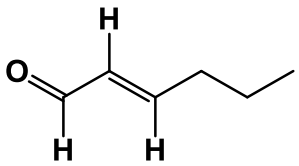
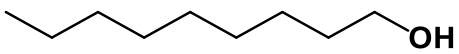
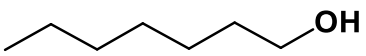
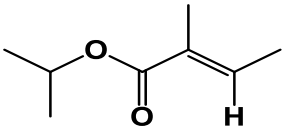
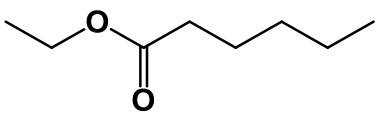
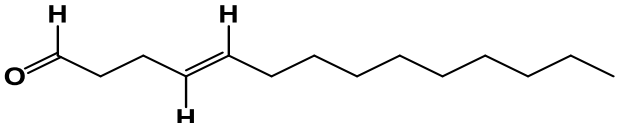
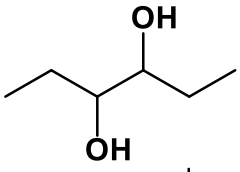
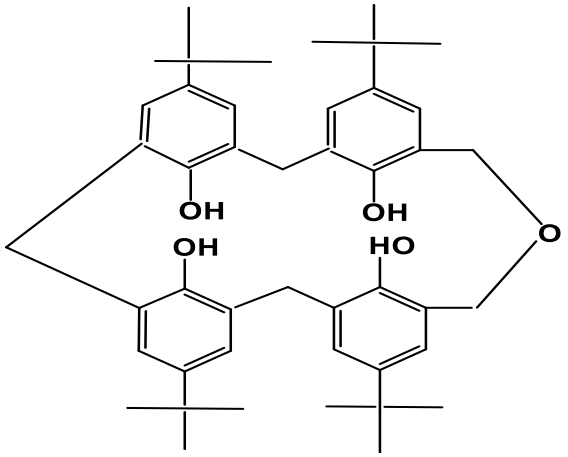
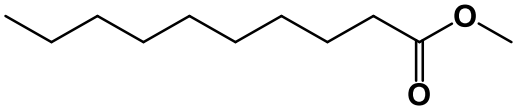
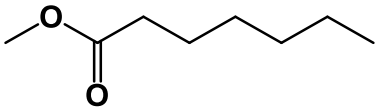
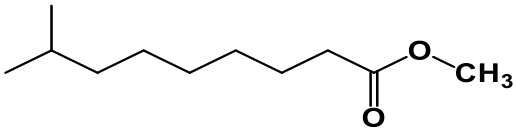
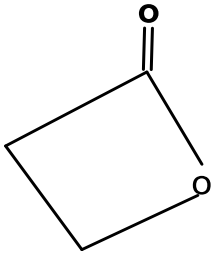
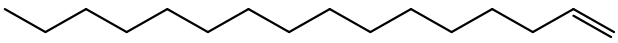
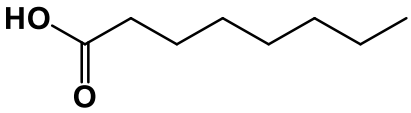
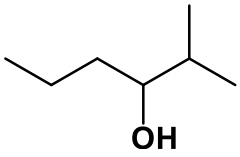
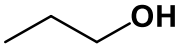
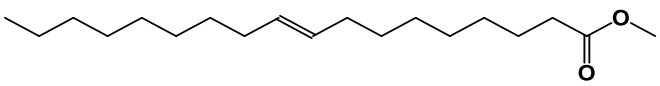
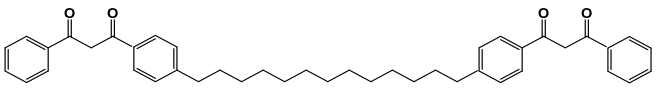
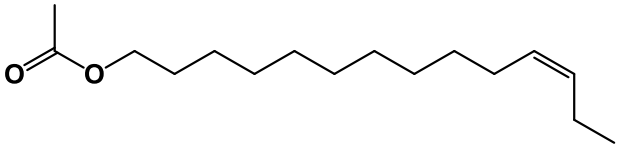
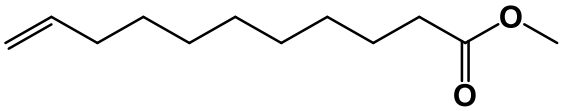


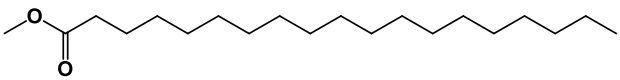
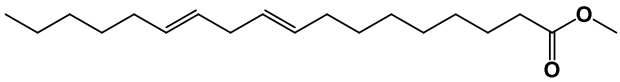
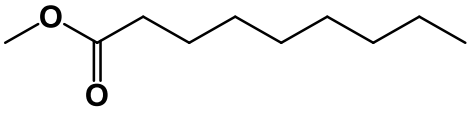
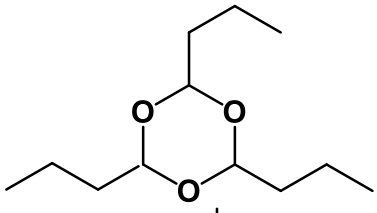
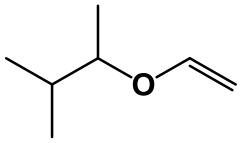
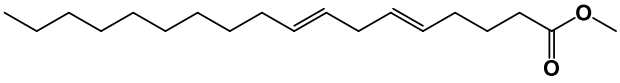
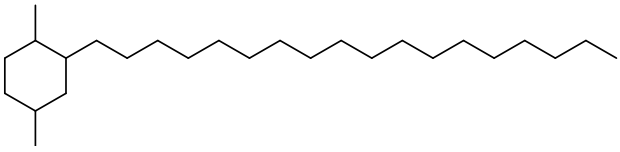
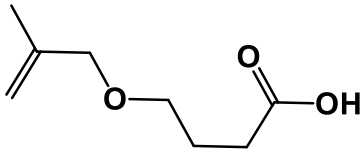
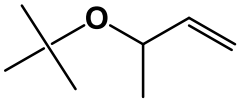
Fig. 6. GC-MS analysis of methanol extract of *Desmodium tortuosum*.

Table 2S: Chemical constituents identified in the unsaponifiable matter of *Desmodium tortuosum* using GC-MS analysis

NO	M.W.	M.F.	R.T.	Area %	Name	Structure
1	98	$C_6H_{10}O$	3.57	0.17	Hexanal<2->	
2	128	$C_9H_{20}O$	4.45	0.77	Nonane<N->	
3	116	$C_7H_{16}O$	6.1	22	Heptanol<N->	
4	142	$C_8H_{14}O_2$	6.23	0.19	Isopropyl Tiglate	
5	144	$C_8H_{16}O_2$	6.97	0.78	Ethyl Hexanoate	

NO	M.W.	M.F.	R.T.	Area %	Name	Structure
6	156	$C_{11}H_{24}$	10.6	0.44	Undecane <N->	
7	704	$C_{33}H_{36}O_{17}$	10.14	0.16	6-C-Xylosyl-8-Cgluco-Sylapigenin permethylated derivative	
8	182	$C_{12}H_{22}O$	20.94	0.26	4-Dodecen-1-aL	
9	118	$C_6H_{14}O_2$	25.58	0.17	Meso-3,4-Hexanediol	
10		$C_{45}H_{58}O_5$	26.70	0.19	7,13,19,25-Tetratertbutyl27,28,29,30 etrahydroxy 2,3-bishomo-3-O- -Xacalix [4] arene	
11	186	$C_{11}H_{22}O_2$	27.08	0.55	Decanoic acid, methyl ester	
12	144	$C_8H_{16}O_2$	28.81	0.22	Heptanoic acid, methyl ester	
13	186	$C_{11}H_{22}O_2$	29.15	0.39	Methyl 8-methylnonanoate	

NO	M.W.	M.F.	R.T.	Area %	Name	Structure
14	72	$C_3H_4O_2$	29.33	0.17	Beta-PROPIOLACTONE	
15	664	$C_{36}H_{56}O_{11}$	30.34	0.20	Tetra-acetyl derivative Of- 25-hydroxybrassinlide	
16	224	$C_{16}H_{32}$	31.13	0.22	Hexadecene <1->	
17	144	$C_8H_{16}O_2$	32.52	0.78	Octanoic acid	
18	116	$C_7H_{16}O$	32.66	0.19	3-Hexanol,2-methyl	
19	60	C_3H_8O	32.77	0.30	1-Propanol	
20	296	$C_{19}H_{36}O_2$	33.03	0.63	9-Octadecenoic acid (Z),methyl ester	
21	628	$C_{43}H_{48}O_4$	33.78	0.18	3Hydroxy1(4{13[4(3-hydroxy 3-phenyl acryloyl) phenyl] tridecyl} phenyl)3-phenylprop-2-en1one	
22	254	$C_{16}H_{30}O_2$	34.33	3.31	11-Tetradecer	
23	198	$C_{12}H_{22}O_2$	34.49	25.50	10-Undecenoic acid,methyl ester	

NO	M.W.	M.F.	R.T.	Area %	Name	Structure
24	312	$C_{20}H_{40}O_2$	34.8	2.63	Nonadecanoic acid,methyl ester	
25	294	$C_{19}H_{34}O_2$	35.17	1.13	9,12 Octadecadienoic acid (Z, Z), methyl ester	
26	172	$C_{10}H_{20}O_2$	35.25	0.25	Nonanoic acid, methyl ester	
27	216	$C_{12}H_{24}O_3$	35.32	0.20	2,4,6-Tripropyl1, 3,5-trioxane	
28	102	$C_5H_{10}O_2$	35.60	0.18	Formic acid, 2-methylpropyl ester	
29	294	$C_{19}H_{34}O_2$	35.98	3.07	5,8-Octadecadienoic acid, methyl ester	
30	364	$C_{26}H_{52}$	36.55	0.29	Cyclohexane,1,4-dimethyl 2-octadecyl	
31	158	$C_8H_{14}O_3$	38.22	0.33	4(2-Methyl-2-propenoxy) butanoic acid	
32	114	$C_7H_{14}O$	38.69	0.52	1-Propene,3 (1,1-dimethyl ethoxy)	

12 Isoprene compounds
1 Diterpene compounds
1 Sesquaterpene compounds
2 Other compounds
Total 41.28%

7 Monoterpene compounds
1 Sestreterpene compounds
1 Tetraterpene compounds
29 Oxygenated compounds

5 Sesquiterpene compounds
1 Triterpene compounds
1 Polyterpene compound
2 Deoxygenated compounds

1- Quantification of total phenolic contents (TPCs)

The results of the total phenolic content showed a remarkable variation among the tested samples, which may be due to the diversity of secondary metabolites in each individual sample. The results were in the following order: [I] fractions (162.63 µg/ml) > [III] fractions (115.70 ± 7.15 µg/ml) > 85% methanol (97.79 ± 6.31 µg/ml) > 90% MeOH (88.15 ± 2.38 µg/ml) > EtOAc (64.74 ± 6.31 µg/ml) > Pet. ether (61.98 ± 4.13 µg/ml) > 70% methanol (60.15 ± 1.88 µg/ml) > n-BuOH (57.75 ± 3.98 µg/ml) (mg GAE/g dry extract). Previous reports showed a highly positive correlation between total phenolic contents and antioxidant activities, reflecting the effective role of phenolic compounds as free radical scavengers [32-34].

2. Antioxidant activity

2.1. Free radical scavenging antioxidant activity using DPPH assay

The oxidative stress associated with generation of destructive effects and harmful health problems as cancer, immunosuppression, inflammation, ischemic heart disease, atherosclerosis, aging, diabetes, and Alzheimer's disease [35-36]. Therefore, the naturally occurring compounds exhibit a strong antioxidant potential due to their abilities to mask the reactive species. Different extracts of *Desmodium tortuosum* were evaluated for their free radical scavenging antioxidant activity using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay. The results revealed that the antioxidant activities (expressed as SC_{50} values in µg/ml) were arranged in the following order; 90% MeOH (1.12 ± 2.36 µg/ml) > CH_2Cl_2 (2.12 ± 4.08 µg/ml) > Pet. ether (2.53 ± 4.08 µg/ml) > *n*-BuOH (2.93 ± 4.09 µg/ml) > EtOAc equal to Fraction [III] (3.46 ± 4.08 µg/ml) > Fraction [I] (3.76 ± 6.22 µg/ml) in comparison with ascorbic acid as standard with SC_{50}= 7.50 µg/ml. Moreover, numerous studies have demonstrated that the high activity antioxidant maybe due to the presence of a complex pattern of the bioactive polyphenolic compounds [37-38].

3. Cytotoxicity

3.1. Brine shrimp lethality bioassay test

The BLST assay is a quick and inexpensive method used to evaluate the lethality of various extracts, fractions or compounds [39]. The results present revealed that the cytotoxic activities of the tested samples are in the order: *n*-BuOH (LC_{50}= 310), EtOAc (LC_{50}= 350), 70 % (LC_{50}= 380). Methanol extracts showed very week activity. According to the global guidelines, values above 1000 µg/ml are considered nontoxic, from 1000 to 500 µg/ml are weakly toxic, and that below 500 µg/ml are toxic [40].

4. HPLC-fingerprint analysis

HPLC-fingerprint analysis is a simple and quick technology widely used to identify the chemical components of plant extracts compared to standard materials [41-42]. Ethyl acetate and two fractions of n-butanol extracts [(I) and (III) &] of D. tortuosum leaves were subjected to chemical profiling through the HPLC-fingerprint technique to identify their chemical ingredients and to characterize the available classes of secondary metabolites [43]. In this study, fourteen standard phenolics were used as reference compounds, including gallic acid (1), chlorogenic acid (2), catechin (3), caffeic acid (4), syringic acid (5), rutin (6), ellagic acid (7), coumaric acid (8), vanillin (9), ferulic acid (10), naringenin (11), propyl gallate (12), quercetin (13), and cinnamic acid (14). The obtained results revealed the variable amounts of the above-mentioned standard compounds in the tested samples. Quercetin, syringic acid, rutin, and cinnamic acid were detected as the major ingredients. On the other hand, coumaric acid, ellagic acid, vanillin, and naringenin were detected as minor components in the ethyl acetate extract. Both chlorogenic acid and ferulic acid are absent. Moreover, quercetin, propyl gallate, cinnamic acid, and gallic acid were recognized as major constituents in fraction [III]. Caffeic acid, coumaric acid, vanillin, and ellagic acid were observed as minor ingredients in the n-butanol fraction [III], and the absence of catechin, rutin, ferulic acid, and naringenin (Table 6 and Figure 4). Also, quercetin, chlorogenic acid, naringenin, gallic acid, and rutin were detected as major constituents in the methanolic fraction [I], where cinnamic acid, ferulic acid, and coumaric acid were detected as minor components, and the absence of catechin and ellagic acid (Table 3 & Figure 4).

Numerous reports stated that there are strong positive relations between the amounts of phenolic compounds in the tested samples and their bioactivities. Due to the unique chemical skeleton and heavy hydroxylation patterns of phenolic compounds, these compounds act as potent free radical scavengers [43-49].

GC-MS analysis

The chemical composition of the ethyl acetate extract of Desmodium tortuosum indicates the presence of thirty-three compounds, representing 89.44% of the total extract composition. These compounds were identified qualitatively based on their retention times and mass spectral fragmentation patterns. They are mainly categorized into fifteen oxygenated compounds (45.4%) and eighteen non-oxygenated compounds (54.54%), classified into six groups: one saturated aliphatic compound (3%), three unsaturated aliphatic compounds (9%), seven oxygenated aliphatic compounds (21%), eighteen aromatic compounds attached to an aliphatic chain (54%), three sesquiterpene compounds (9%), and one anthraquinone compound (3%). The major identified compounds include Benzene (1-butyloctyl) (11.88%), Himachalene <α-> (11.08%), Benzene (1-pentyl heptyl) (9.96%), Undecanal, 3-phenyl (8.21%), Phenyl Ethyl Alcohol (7.43%), Benzene (1-ethyldecyl) (6.50%), Benzene (1-methyldecyl) (5.78%), and Benzyl Butyrate (5.31%).

Qualitative GC-MS analyses of the unsaponifiable matter of D. tortuosum resulted in the identification of thirty-two compounds (Table 5), representing 41.19% of the total extract composition. These compounds were identified qualitatively based on their retention times and mass spectral fragmentation patterns. They are categorized into twenty-seven oxygenated compounds (84%) and five deoxygenated compounds (15%). All compounds are classified into six groups: twenty-five oxygenated aliphatic compounds (78%), one saturated aliphatic compound (3%), one unsaturated aliphatic compound (3%), one flavonoid derivative compound (3%), one steroid derivative compound (3%), and three aromatic compounds conjugated with an aliphatic chain (9.3%). The major identified compounds include 10-Undecenoic acid, methyl ester (25.50%), 5,8-Octadecedienoic acid methyl ester (3.07%), and Nonadecanoic acid methyl ester (2.63%).

The data for both extracts indicate that D. tortuosum is rich in different types of aliphatic fatty acids. Most of the identified compounds are cited in the literature for their various biological activities. For example, 1-Octadecene has shown antibacterial, antioxidant [50], and anticancer effects [51]. Dodecanoic acid methyl ester exhibited antibacterial, antiviral, and antifungal properties [52], while 1-Hexadecene demonstrated antibacterial, antifungal [53-55], and antioxidant activity [56]. 9-Octadecenoic acid (Z) methyl ester has been reported for its antioxidant and anticancer effects [41-57]. The major identified compound Benzene (1-butyloctyl) has antimicrobial activity [58].

CONCLUSION

Different extracts of D. tortuosum were prepared and evaluated for their biological and chemical characteristics. Among all the tested extracts, the 90% methanol extract showed the highest antioxidant activity. On the other hand, the cytotoxic test showed weak toxic effects, with the most active extract being n-BuOH (LC_{50}= 310). Regarding its chemical content, HPLC-Fingerprint Analyses indicated the presence of phenolic compounds in both the ethyl acetate extract and the two butanol fractions, which are responsible for antioxidant activity. Additionally, GC-MS analysis determined the presence of many fatty acids and other aliphatic compounds with a variety of biological activities. The plant is considered a candidate for further chemical and pharmaceutical investigations.

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السمية الخلوية، الأنشطة المضادة للأكسدة، تحليلات GC / MS وHPLC لمستخلصات ديسموديوم تورتوسوم DC (Sw.)

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ملخص

تعد عائلة Fabaceae ثالث أكبر عائلة نباتية مزهرة، وقد أظهر جنس ديسموديوم مدى واسعًا في الأنشطة البيولوجية ومجموعة متنوعة من المكونات الكيميائية. تم تقييم انشتطها السامة. فى الدراسة الحالية ل ديسموديوم تورتوسوم تم اختبار مستخلصات مختلفة منه لتقييم انشتطها السامة للخلايا وكمضادات اكسدة وتقدير المحتوى الفينولى الكلى. تم إجراء الأنشطة المضادة للأكسدة بشكل كمي باستخدام الشقوق الحرة 1.1'- ثنائي فينيل -2-بيكرايل هيدرازيل (DPPH) ولقد تم تقييم النشاط السام للخلايا عن طريق اختبار اماتة يرقات الجمبري الملحي .(BSLT) أظهرت نتائج النشاط المضاد للأكسدة أن فعالية الكسح الجذري لـ DPPH (SC50) تراوحت من 1.12 إلى 61.22 ميكروغرام / مل بالمقارنة بحمض الأسكوربيك (SC50 = 7.45 ميكروغرام / مل)، من بين جميع الأجزاء المختبرة وجد أن 90٪ الميثانول هو الأكثر نشاطًا. من ناحية أخرى ، وجد أن الأنشطة السامة لمستخلص البيوتانول على الخلايا هو الأكثر سمية بالمقارنة بالمستخلصات الأخرى. تم إجراء تحليلات كروماتوجرافيا سائلة عالية الأداء (HPLC-fingerprint) لتقدير التركيب الكيميائي ونسبة المركبات الفينولية ، وقد حدد تحليل كروماتوغرافيا الغاز – مطياف الكتلة (GC-MS) وجود الأحماض الدهنية والمركبات الأخرى. المركبات الرئيسية التي تم تحديدها هي البنزين (1-بيوتيلوكتيل) (11.88٪) وهيماشالين <11.08) <-α ٪ (لمستخلص أسيتات الإيثيل و 10-حمض أونديسينويك ، إستر الميثيل (25.50٪)، للمواد غير السائلة.

الكلمات الدالة: مضادات الأكسدة، السمية الخلوية، ديسموديوم تورتوسوم، كروماتوجرافيا الغاز، مطياف الكتلة، تحليلات كروماتوجرافيا سائلة عالية الأداء.

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Formulation, Development, and Evaluation of Bosentan Monohydrate Spray Dried- Solid Dispersion Tablets for Improved Dissolution Profile

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ABSTRACT

Bosentan monohydrate (BM) is utilized for the treatment of pulmonary arterial hypertension, exhibiting poor aqueous solubility and bioavailability. This study aims to enhance the dissolution rate of the drug using Eudragit®EPO through spray drying. The drug and Eudragit®EPO were combined in ratios of 1:1, 1:2, 1:3, 1:4, and 1:5 (w/w) to generate compositions SD1 to SD5. SD5, at a 1:5 drug-to-carrier ratio, demonstrated a statistically significant increase in saturation solubility and drug content. Six tablet formulations (F1 to F6) containing SD5 and tableting excipients were developed and processed. Formulation F2, consisting of 26.36% HPMC K4M and 23.63% MCC, exhibited the highest dissolution and drug release. The probable mechanism underlying BM dissolution in SD involves its amorphous form and the solubilizing effect facilitated by hydrogen bonding between BM and Eudragit®EPO. The carrier's binding effect likely contributed to high tensile strength, low friability, and extended disintegration time. Direct mixing of SD with HPMC might have improved the uniformity of SD within the tablet matrix and the release profile. This study demonstrates the efficacy of spray drying in preparing SD of BM with Eudragit®EPO, potentially enhancing its solubility and stability.

Keywords: Bosentan monohydrate, Eudragit®EPO, Solubility, Dissolution, Solid-dispersion, Spray drying.

1. INTRODUCTION

It is commonly recognized that approximately 40% of recently developed medications exhibit low water solubility, compromising absorption and increasing gastro mucosal toxicity. Three primary criteria affecting a drug's bioavailability include its solubility, permeability, and dissolution [1]. According to the Biopharmaceutical Classification System (BCS), drugs are categorized based on their solubility and permeability [2]. Drugs with poor solubility belong to BCS class II and IV. The poor solubility of drugs poses a challenge to bioavailability. In such cases, strategies such as particle size reduction, polymorphism, molecular encapsulation, incorporation of surface-active agents, and solid dispersion (SD) can be employed to enhance solubility [3]. In the case of SD, the carrier component dissolves upon exposure to aqueous solutions, releasing drug molecules as tiny, dispersed particles [4]. The bioavailability of weakly water-soluble drugs is ultimately improved due to increased surface area and dissolution rate. By reducing particle size and increasing particle porosity, the drug in a soluble hydrophilic carrier enhances the rate of dissolution [5]. Therefore, enhancing the bioavailability of these medications and minimizing their negative effects by altering their drug release profile is conceivable.

SD is a prominent technique used for dissolution rate and solubility enhancement, as well as for improving the oral absorption of weakly water-soluble drugs [2]. SD is a composite mixture prepared by mixing one or more solid

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particles and dispersing them into an inert medium through fusion, solvent evaporation, or the melting method. SD contains a hydrophilic matrix for solubility enhancement and a hydrophobic drug whose solubility is to be improved, wherein the matrix can be either crystalline or amorphous [6]. SD was originally used to create a eutectic combination of pharmaceuticals with water-soluble carriers to overcome the limited bioavailability of lipophilic drugs [7,8]. Spray drying is a superior method compared to other methods as it operates at comparatively low temperatures, preventing most decomposition that occurs during the fusion process in the melting method. Spray drying has no solvent limitations and is most suitable for large-scale production [9]. Previously, SD of BM was made using hydroxypropyl β-cyclodextrin (HP β-CD) and polyethylene glycol (PEG) 4000 polymers, but BM in such SD retained its crystallinity, limiting its solubility [10]. Additionally, PEG 4000 exhibits an uncontrolled rate of hydration, rheological changes during shelf-life, and the possibility of microbial contamination during use [6].

Eudragit® EPO is a cationic polyelectrolyte belonging to methacrylate copolymers. It is composed of dimethylaminoethyl methacrylate, butyl methacrylate, and methyl methacrylate in a molar ratio of 2:1:1 [11]. It carries a positive charge, so when dissolved in an aqueous medium, it forms a solid dispersion (SD) with a drug that has a negative charge [12]. Samples containing coated drug particles, as well as reference formulations with an equivalent amount of this polymer, exhibited higher tensile strength, lower friability, and longer disintegration times [11]. These outcomes result from the binding effect exhibited by Eudragit® EPO. Additionally, a modified processing method, in which the molten drug-carrier mass was directly mixed with hydroxypropyl methylcellulose (HPMC), improved the uniformity of the drug in the tablet matrix and the release profile [13].

Bosentan monohydrate (BM) is used to treat pulmonary artery hypertension (PAH) and possesses dual endothelin receptor antagonistic characteristics [14]. The structure of BM is shown in Figure 1.

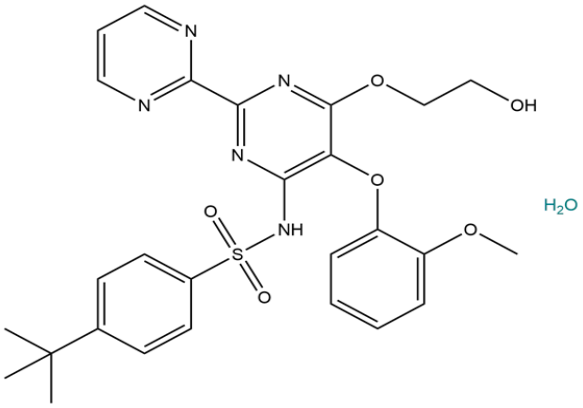

Figure 1. Structure of Bosentan Monohydrate

BM is prescribed at a daily dose of 125–250 mg for treating pulmonary arterial hypertension (PAH). It exhibits a plasma peak concentration between 3-5 hrs after administration, with a biological half-life of 5.4 hrs [15]. The oral bioavailability of BM is 50%, with variability in its absorption due to its poor aqueous solubility. The aqueous solubility of BM is 1 mg/100 mL, categorizing it as BCS class II [16]. Hence, the present study aimed to formulate BM-loaded Eudragit® EPO solid dispersion (SD) microparticles with the primary objective of

achieving rapid drug release in the acidic pH of the stomach. To enhance solubility, SD was attempted by spray drying using polymers along with hydroxypropyl β-cyclodextrin (HP β-CD). Additionally, efforts were made to formulate tablets of SD with hydroxypropyl methylcellulose (HPMC) K4M, microcrystalline cellulose (MCC) as binders, and talc and magnesium stearate as glidant and lubricant to create an improved dissolution profile for therapeutic effectiveness.

2. RESULTS AND DISCUSSION

Particles produced through spray drying have smaller sizes, enhancing their surface area and expediting the dissolution process.

The carrier incorporated in the solid dispersion (SD) significantly improves the drug's solubility through enhanced particle wettability, thereby increasing the drug's bioavailability. Drugs formulated as SD exist as supersaturated solutions in metastable polymorphic forms, leading to increased solubility and availability in the amorphous state [17, 18, 19]. Therefore, SD of the poorly soluble Bosentan monohydrate (BM) was formulated to enhance its solubility.

The well-known gastro-soluble polymer Eudragit® E100 is available in powder form as Eudragit® EPO. Tertiary amino groups in the polymer ionize at an acidic pH, making Eudragit® E100 soluble in gastric fluid when the pH is lower than 5. Researchers have utilized Eudragit® E100 to prepare SD of hydrophobic drugs with dissolution rate-dependent bioavailability due to its excellent solubility in gastric fluids [20].

2.1 Preparation and optimization of SD

Improved bioavailability results from the increased surface area achieved through small particle size. Spray drying was employed to produce SD, as it allows for rapid solvent evaporation, quickly converting the drug-carrier solutions into solid particles. The prepared SD was further evaluated for the following parameters.

2.2 Evaluation of SD powder:

SD compositions (SD1 to SD5) were optimized by evaluating the prepared SD for drug content and saturation solubility.

2.2.1 Flow properties and Heckel plot of SD powder:

The solid dispersion (SD) compositions were evaluated for various flow properties, including the angle of repose, Hausner's ratio, and Carr's index. The results are displayed in the table below. The SD5 composition exhibits an angle of repose of 27.32±0.2°, Hausner's ratio of 1.18±0.85, and Carr's index of 14.7±0.47%, indicating good flow and compressibility for the SD5 composition [21].

Table 1. Flow properties of BM SD

Batch code	Angle of Repose (°)	Bulk density (g/cm^3)	Tap density (g/cm^3)	Hausner's ratio	Carr's index (%)
SD1	42.40±0.3	0.4718±0.019	0.6178±0.021	1.31±0.16	23.6±0.41
SD2	46.10±0.6	0.4627±0.017	0.5547±0.042	1.20±0.21	16.6±0.11
SD3	38.20±0.5	0.4579±0.036	0.5741±0.063	1.25±0.42	20.2±0.17
SD4	48.19±0.9	0.4905±0.085	0.5853±0.051	1.19±0.57	16.2±0.58
SD5	27.32±0.2	0.4375±0.074	0.5131±0.011	1.18±0.85	14.7±0.47

(n= 3, mean±SD)

The Heckel plot for the BM-loaded Eudragit® EPO solid dispersion (SD) exhibited a Type C curve, indicating particle rearrangement and fragmentation of large aggregates under low compressional pressure. As the compression force increased, the curves became linear due to plastic deformation. It can be inferred that the curved

region at low pressures is associated with individual particle movement in the absence of interparticle bonding. The transition from curved to linear corresponds to the minimum pressure required to form a coherent compact, indicating the formation of coherent compacts in this region.

Table 2. Heckel plot of BM SD

Pressure (Tons)	Thickness (cm)	Diameter (cm)	Radius (cm)	Weight of tablet (mg)	Volume (πr^2h)	Density (mg/ml)	Hardness (Kg/cm^2)	Relative density	1/1-RD	In (1/1-RD)
0.5	0.31±0.3	0.8	0.4	200±0.1	0.1557±0.4	1284.52±0.7	4±0.1	0.7739	4.42	1.4861
1	0.30±0.1	0.8	0.4	200±0.7	0.1507±0.2	1327.14±0.1	5±0.3	0.7996	4.99	1.6074
1.5	0.29±0.2	0.8	0.4	200±0.5	0.1456±0.1	1373.62±0.2	7±0.2	0.8276	5.80	1.7578
2	0.27±0.5	0.8	0.4	200±0.4	0.1356±0.3	1474.92±0.1	8±0.4	0.8886	8.97	2.1938
2.5	0.24±0.4	0.8	0.4	200±0.6	0.1205±0.6	1659.75±0.5	9±0.5	1	-	
3	0.24±0.6	0.8	0.4	200±0.2	0.1205±0.5	1659.75±0.4	9±0.1	1	-	

(n= 3, mean±SD)

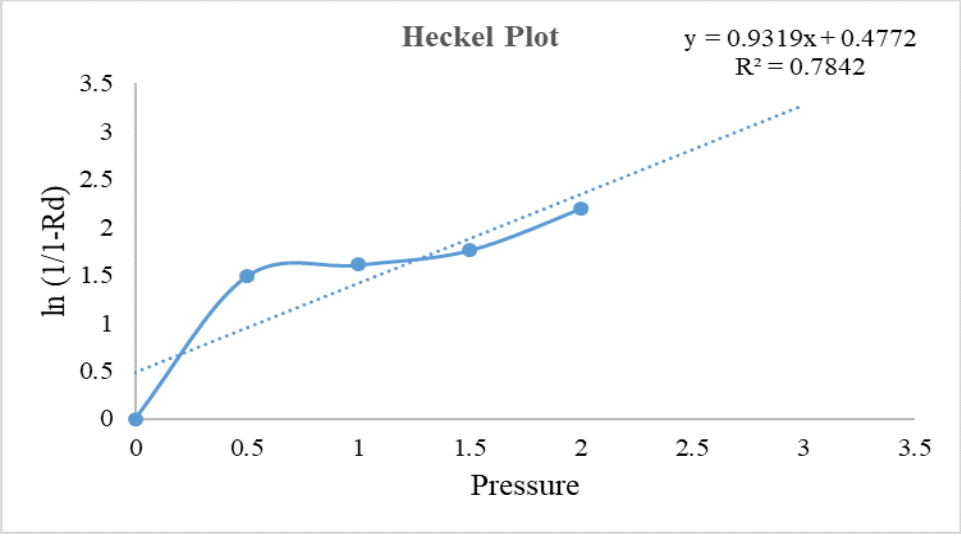

Figure 2. Heckel plot of BM-loaded Eudragit® EPO SD

2.2.2 Scanning Electron Microscopy (SEM):

SEM studies were conducted to qualitatively examine the shape and surface morphology of the BM-loaded Eudragit® EPO solid dispersion (SD), and the obtained images are presented in Figure 3. According to Lee H et al., the pure bosentan drug exhibited irregular, non-spherical morphology, a polydisperse size range, and a crystalline particle state. In contrast, the BM-loaded Eudragit® EPO SD revealed irregularly folded and flocculated round morphology with a smooth surface and monodisperse size range.

The differences in particle morphology between the pure drug and the spray-dried formulations suggested the conversion of the drug from a crystalline form to an amorphous form, consistent with the XRPD results [22].

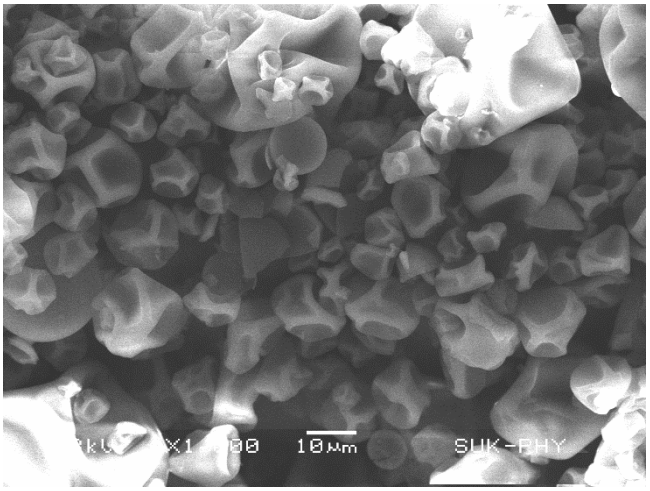

Figure 3. SEM of BM-loaded Eudragit® EPO SD

2.2.3 Particle size:

The particle size of the spray-dried dispersions is presented in Table 3. The SD5 composition has the smallest particle size of 213.4 nm, which enhances the surface area of smaller particles, promoting better wetting and faster dissolution. This, in turn, leads to improved drug solubility and bioavailability. Consequently, SD5 will be utilized for further formulation [22].

Table 3. Particle size of BM SD5

Batch code	Particle Size (nm)
SD5	213.4±0.6

(n= 3, mean±SD)

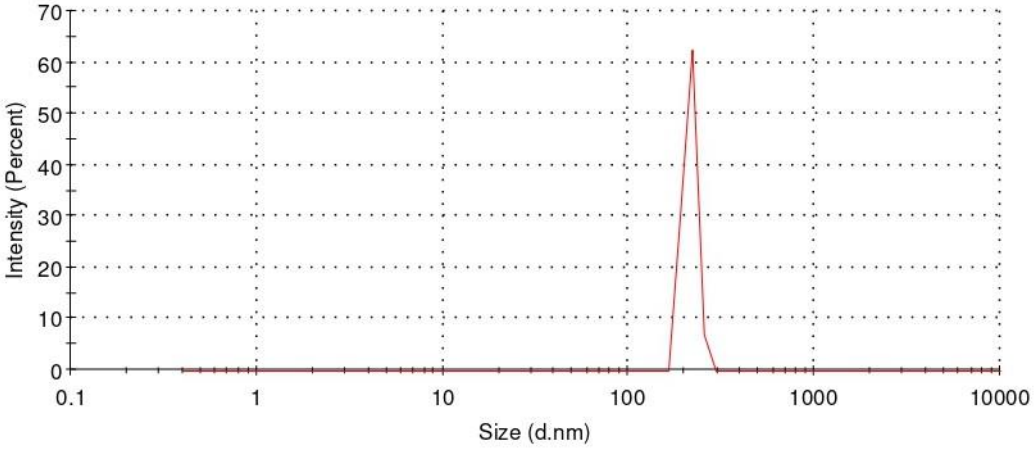

Figure 4. Particle size of BM-loaded Eudragit® EPO SD5

2.2.4 Drug content:

The spray-dried dispersions were solid, free-flowing, fine powder with a drug content of 74.2% and 94.4%, as shown in Table 4. SD5 exhibited a drug content of 94.4 ± 0.9% with uniform drug distribution, good flowability, and compressibility, as observed in the flow properties.

2.2.4 Saturation solubility:

Saturation solubility showed statistically significant improvements in solubility, increasing from 55.54±0.21 to 138.16±0.47 µg/mL. BM was formulated into SD employing a carrier Eudragit® EPO at a 1:5 ratio, as detailed in Table 4. In SD, the saturation solubility of BM increased to 138.16±0.47 µg/mL. Based on the overall evaluation, especially the saturation solubility and drug content, the composition SD5 was selected for preparing tablet formulations.

Table 4. Drug content and saturation solubility of BM SD

Batch code	BM : Eudragit® EPO ratio	Solubility in water (mg/mL)	Drug content (%)	Saturation solubility (mg/mL)
Pure drug	-	0.009	-	50.61±.043
SD1	1:1	0.085	74.2±0.5	55.54±.021
SD2	1:2	0.091	82.7±0.7	88.72±0.19
SD3	1:3	0.098	83.9±0.4	104.33±0.36
SD4	1:4	0.099	91.5±0.1	123.63±.058
SD5	1:5	0.103	94.4±0.9	138.16±0.47

(n= 3, mean±SD)

2.3 Formulation of tablets:

The composition SD5 was selected for the formulation of tablets among various compositions. The wet granulation technique was employed in this instance for tablet creation. HPMC K4M and MCC were used as binders, while talc and magnesium stearate served as glidant and lubricant. The choice of HPMC K4M was based on its ease of usage, better compressibility, high drug loading capacity, good stability over a wide pH range, and quick swelling [23]. MCC was selected for its ability to hold water, aiding in uniform granulation [24]. Talc was included to reduce interparticulate friction, van der Waals forces, and electrostatic charges, improving powder flow properties [25]. Magnesium stearate, an affordable and chemically stable lubricant, was used for its high lubrication power and high melting point [26]. Six tablet formulations, F1 to F6, were designed using SD5 and other tableting excipients, as detailed in Table 5.

Table 5. The formulation composition of BM SD tablets

Compoenents (mg)	F1	F2	F3	F4	F5	F6
SD5 powder	90	90	90	90	90	90
HPMC K4M	56.74	52.73	48.75	44.74	40.73	37.75
MCC	43.26	47.27	51.25	55.26	59.27	62.25
Talc	7	7	7	7	7	7
Magnesium stearate	3	3	3	3	3	3
Total weight	200.0	200.0	200.0	200.0	200.0	200.0

2.4 Evaluation of Tablets:

Six tablet formulations (F1-F6) were prepared and evaluated to determine an optimized tablet formulation.

2.4.1 Thickness:

Tablet thickness is crucial for administration comfort and therapeutic efficacy [27]. It influences disintegration and dissolution behavior. The thickness of tablet formulations (F1-F6), measured by digital vernier caliper, ranged from 2.85±0.42 to 3.92±0.05 mm, as shown in Table 6. This falls within the pharmacopoeial limit for thickness, which specifies that the tablet diameter should be 8 mm or less.

2.4.2 Hardness:

Tablet hardness should be within an optimal range, as both excessively high and low hardness can impact disintegration. The hardness of the tablets was determined using the Monsanto hardness tester. The hardness of all tablet formulations was in the range of 3.7±0.47 to 5±0.31 kg/cm2, as detailed in Table 6. This falls within the pharmacopoeial specifications of 3-6 kg/cm^2.

2.4.3 Friability:

Tablet friability measures the resistance of compressed tablets against coating and packaging during manufacturing and shipping. The pharmacopoeial limit for friability is less than 1% of the tablet mass. The % loss of tablets for formulations F1-F6 ranged from 0.38±0.02 to 0.54±0.03%, as indicated in Table 6. The friability test results suggest compliance with the official limits, ensuring sufficient tablet strength.

2.4.4 Weight variation:

Tablet weight variation is crucial for verifying dosage consistency and supporting tablet strength, safety, and identity. The weight variation of tablets in each batch was assessed using an analytical balance, as detailed in Table 6. The % weight variation for batches F1-F6 ranged from 4.18±0.07 to 7.54±0.8%, in compliance with pharmacopoeial specifications of ±7.5%.

2.4.5 Disintegration time:

Disintegration time reflects the duration needed to break down a tablet into smaller pieces, creating a larger surface area for faster dissolution. In vitro disintegration time was determined using a disintegration tester, and the results for formulations F1 through F6 ranged from 32±5 to 45±1 sec, as presented in Table 6. These values align with pharmacopoeial standards of 3 minutes.

2.4.6 Drug content:

The drug content of all prepared tablet formulations was assessed using a UV-visible spectrophotometer. The drug content in formulations ranged from 93.15±0.2 to 95.41±0.05%, as shown in Table 6. Formulation F2 exhibited the highest drug content at 95.41±0.05%, surpassing the values obtained for other formulations.

Table 6. Evaluation of bosentan tablets

Batch code	Thickness (mm)	Hardness (kg/cm^2)	Friability (%)	Weight variation (%)	Disintegration time (sec)	Drug content (%)
F1	2.91±0.21	4.5±0.23	0.39±0.05	4.56±0.10	36±2	93.55±0.21
F2	3.92±0.05	5.0±0.31	0.54±0.03	7.54±0.8	45±1	95.41±0.05
F3	2.85±0.42	4.4±0.73	0.38±0.02	5.22±0.12	38±3	94.85±0.75
F4	3.75±0.81	4.5±0.99	0.45±0.08	4.18±0.07	40±4	93.15±0.12
F5	2.94±0.58	3.9±0.93	0.39±0.09	3.17±0.5	33±6	94.32±0.32
F6	2.86±0.65	3.7±0.47	0.45±0.04	2.45±0.20	32±5	94.62±0.89

(n= 3, mean±SD)

Based on the data analysis of the evaluated tablet characteristics, including a thickness of 3.92±0.05 mm, hardness of 5.0±0.31 kg/cm2, friability of 0.54±0.03%, weight variation of 7.54±0.8%, disintegration time of 45±1 sec, and drug content of 95.41±0.05%, Formulation F2 was concluded to be the optimized one.

2.4.7 *In vitro* drug release:

In vitro drug release from plain BM and the optimized F2 BM SD was studied using USP XXII type II dissolution test equipment. The results of in vitro drug release were plotted as cumulative percent drug release vs. time, as illustrated in Fig. 1. At the end of 9 hours, Formulation F2 exhibited the highest dissolution rate of 98.21%, while for the same duration, the release from plain BM was 38.7%. Comparisons with previously reported work concluded that BM could be successfully formulated with HPMC K4M and HPMC K15M. Formulation containing BM: HPMC K4M in the ratio 1:0.5 was optimized, maintaining a drug release of 96.3 ± 0.53% for 12 hours. Furthermore, the cumulative drug release obtained through the dissolution study of BM formulated with Eudragit® EPO was 98.21%, sustaining drug release up to 9 hours. Therefore, the BM tablet formulation containing Eudragit® EPO appeared as an effective polymer that enhanced the drug's dissolution ability.

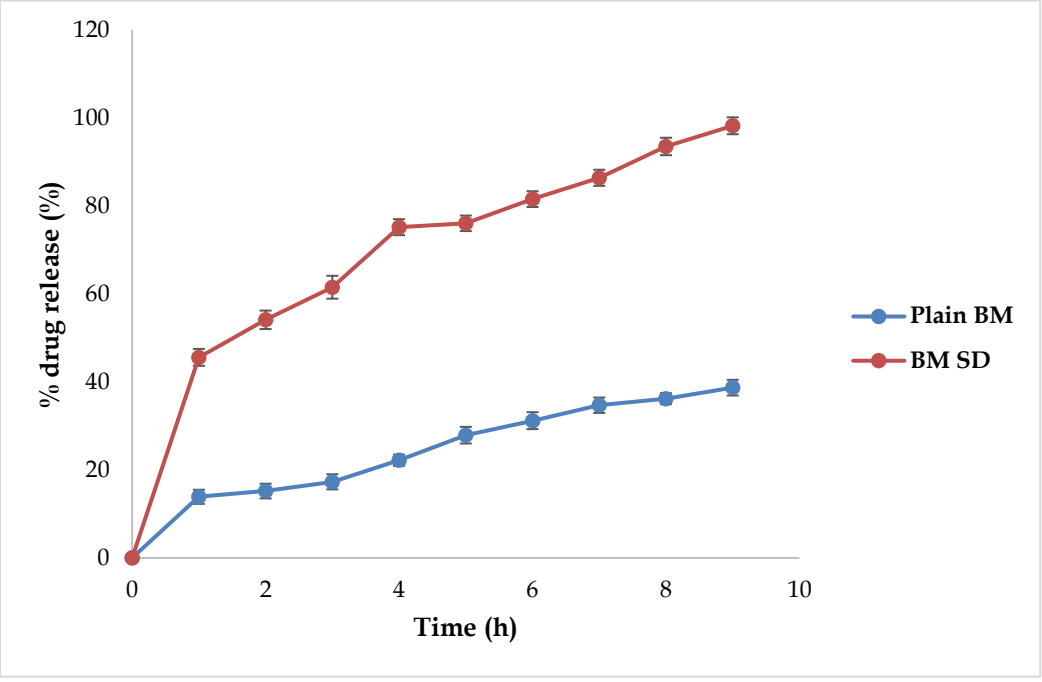

Figure 5.A plot of Cumulative % Drug Release versus Time of Plain BM and BM SD

2.4.8 XRPD Analysis:

The X-ray powder diffraction pattern of pure BM and Formulation F2 is presented in Fig. 2. The drug's diffractogram pattern was indicated by the existence of sharp peaks at 21.8º and 27.8º, confirming its crystallinity. In Formulation F2, in addition to a decrease in the sharpness of peaks, the multiple sharp peaks that were present with the plain drug were statistically decreased, suggesting amorphization of the drug. This physical change demonstrates that the BM was dissolved to a molecular level in a SD, which further controls the drug's ability to effectively dissolve in the medium [28].

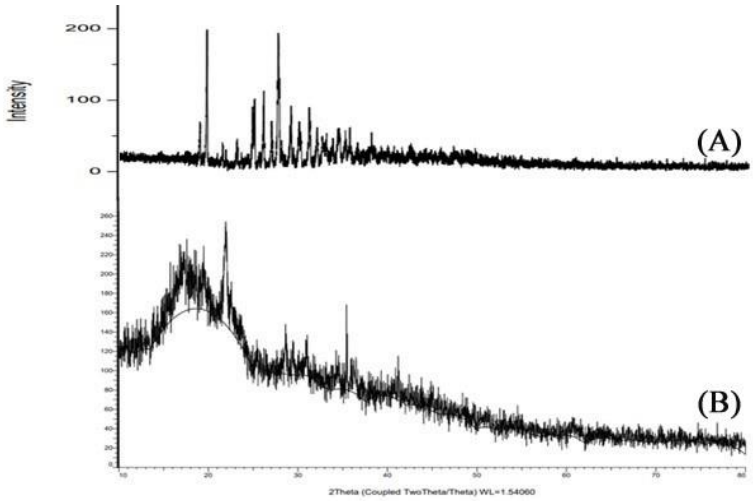

Figure 6. XRPD Analysis of (A) Bosentan monohydrate and (B) F2 formulation

2.4.9 FTIR Analysis:

The FTIR distinctive peaks in BM, Fig. 3, appeared at 2960.73 cm^{-1} for CH_3-stretching, 3493.09 cm^{-1} for OH-stretching, 1080.14 cm^{-1} for C-O stretching in ether, and 1384.89 cm^{-1} for S=O stretching. The CH_3 and OH stretching peaks at 2927 cm^{-1} and 3383 cm^{-1} for formulation F2 showed only a minor change. It reflects intermolecular hydrogen bonding potential of F2 formulation. These findings were close to results reported by Hajare et al. (2020) for clobetasol propionate [29].

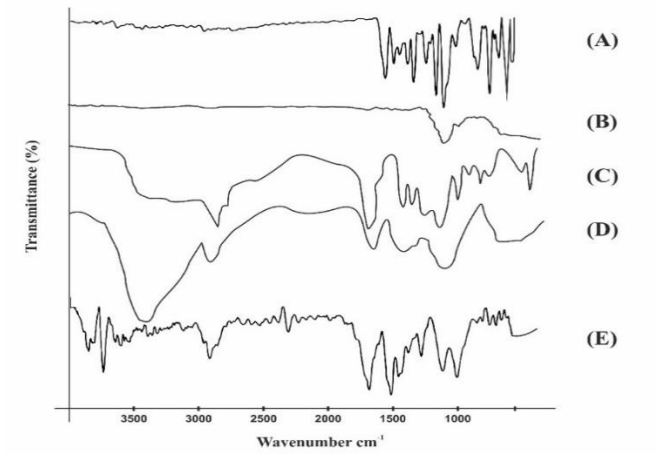

Figure 7. FTIR spectra of (A) Bosentan monohydrate and (B) HPMC K4M (C) Eudragit EPO (D) MCC (E) F2 formulation

2.4.10 DSC Analysis:

A popular thermal analysis method known as differential scanning calorimetry (DSC) provides accurate data on a material's physical and energetic properties. The melting point endotherm of BM was found to be at 104.2 ℃, whereas that of the F2 batch was found at 68.8 ℃. The DSC thermogram of formulation F2 showed reduced and diffused endothermic peaks, as seen in Figure 7. The

diffused DSC pattern of the formulation indicated that the endothermic peak had shifted, and its strength had decreased, indicating that the drug had changed from being crystalline. The drug's dissolving rate statistically increased as a result of its conversion from the crystalline to the amorphous state, as the latter has high internal energy and is thought to be in a highly disordered condition [30].

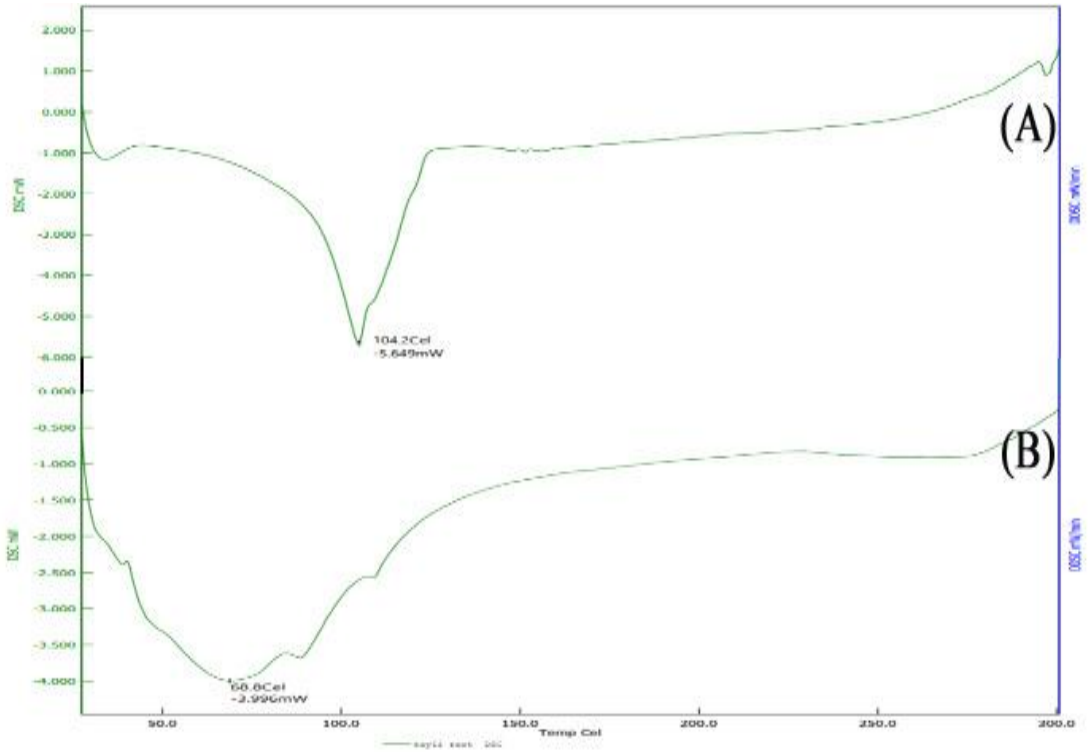

Figure 8. DSC Thermogram of (A) Bosentan monohydrate and (B) F2 formulation

2.4.11 Drug release kinetics study:

To estimate the mechanism of drug release from SD, the in vitro release data was fitted to multiple drug release kinetics models. The findings showed that the formulation was best explained by Higuchi release kinetics (square root kinetics), as depicted in Figure 6, indicating that the drug diffuses at a slower pace as the distance for diffusion increases.

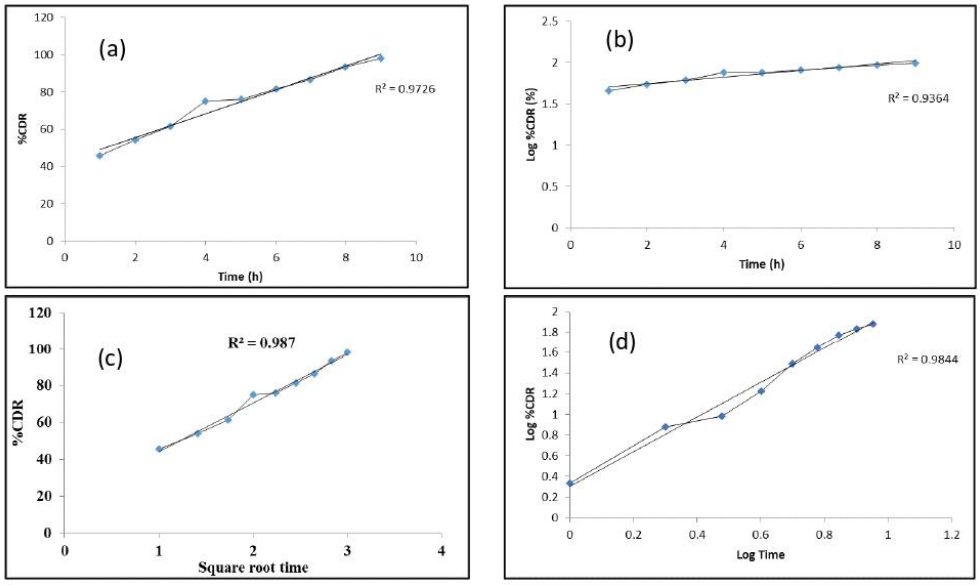

Figure 9. Comparative plots of (a) zero-order release kinetics, (b) first-order release kinetics, (c) Higuchi (SQRT) release kinetics, and (d) Korsemeyer-Peppas model for the selected SD formulation

2.4.12 Stability study:

According to ICH guideline requirements, stability tests of the optimized formulation F2 were performed for three months at 40°C and a relative humidity of 75%. After 90 days, the sample was removed and tested for drug content and percent drug release, as shown in Table 7. At the end of the 3 months, the drug content was within the pharmacopoeial limit (95.32%), indicating that the prepared solid dispersion of BM was stable.

Table 7. Drug content and release data for stability study

Sr. No.	Drug content (%)		Drug release (%)	
	0^{th} Day	90^{th} Day	0^{th} Day	90^{th} Day
1	95.41	95.35	98.21	98.12
2	95.48	95.21	98.30	98.21
3	95.35	95.40	98.28	98.31
Mean	95.41±0.053	95.32±0.080	98.26±0.038	98.21±0.077

(n= 3, mean±SD)

3. CONCLUSION

BM, classified as a BCS Class II drug, poses challenges due to its poor water solubility. Eudragit® EPO, a newly introduced polymer, has been investigated for its ability to enhance solubility and dissolution of BM. The preparation of solid dispersion complexes of the drug with Eudragit® EPO using spray drying has proven effective in enhancing the drug's solubility and dissolution. The likely mechanism behind the improved dissolution of BM involves a physical change in the state of the solid dispersion and the solubilizing effects facilitated by hydrogen bonding formed between Eudragit® EPO and the drug molecules.

Further exploration of Eudragit® EPO in extensive research, encompassing various model drugs, stability studies, and clinical testing, is warranted.

4. MATERIALS AND METHODS

4.1 Materials

Bosentan monohydrate was provided by Megafine Chemicals Pvt. Ltd., Mumbai, India. Evonik Industries Pvt. Ltd., Mumbai, supplied Eudragit® EPO. HPMC K4M was purchased from Loba Chem Ltd., Mumbai, India. Analytical-grade chemicals were utilized for all compounds and reagents.

4.2 Preparation and optimization of solid dispersion:

Solid dispersion (SD) was created via spray drying because it enables speedy solvent evaporation, resulting in a quick transformation of the drug: carrier solution into solid particles. Different drug carrier ratios of BM were dissolved in methanol (10% w/v) with Eudragit® EPO (1:1, 1:2, 1:3, 1:4, and 1:5). To obtain a clear solution, the liquids were thoroughly mixed. Using a spray drier (Labultima, Mumbai, India), the clear solutions were dried at an inlet temperature of 80 °C, output temperature of 55 °C, feed pump rate of 8 mL/min, aspiration rate of 45 mbar, and atomization air pressure of 2.5 kg/cm2. To remove any remaining solvents, the resultant SD was vacuum desiccated for 24 h. The dried bulk was then triturated and passed through mesh 80 to ensure a consistent particle size and kept in a tightly sealed container until it was used [31]. Furthermore, the prepared compositions were evaluated for drug content and saturation solubility to optimize the composition of BM SD.

4.3 Evaluation of prepared solid dispersion:

4.3.1 Flow properties and Heckel plot of SD powder:

A. Angle of repose

The angle of repose is a measurement of a powder's or granules' flowability. The powder is carried freely through the fixed funnel to create a heap of specified height, and the angle formed by the powder with that of the base is determined, from which the flow type of the sample is identified. The powder's angle of repose was calculated using the fixed funnel free-standing cone technique using the formula [21]:

$$\theta = \tan^{-1}\frac{h}{r} \quad \text{……. (Equation 1)}$$

Where, h: Height between the lower tip of a funnel and the base of a heap of granules, r is the radius of the base of heap formed.

B. Bulk density

The bulk density of all batches of S-SNEDDS was assessed by gently pouring 5g of powder through a glass funnel into a 10 mL graduated cylinder and recording the volume filled by the sample. The following formula was used to compute the bulk density [21]:

$$\text{Bulk density}\left(\frac{g}{mL}\right) = \frac{\text{Weight of samples in g}}{\text{Volume occupied by sample}} \quad \text{(Equation 2)}$$

C. Tapped density

A glass funnel was used to pour 5 g of powder into the 10 mL graduated cylinder. A consistent volume was attained by tapping the cylinder from a distance of 2 inches. After recording the volume of powder occupied after tapping, the tapped density was obtained as follows [21]:

$$\text{Tapped density}\left(\frac{g}{mL}\right) = \frac{\text{Weight of samples (g)}}{\text{Tapped volume occupied by sample}} \quad \text{(Equation 3)}$$

D. Hausner's ratio

The Hausner ratio was calculated to characterize the flow of a powder blend. When the Hausner ratio exceeds 1.25, it is regarded to be a sign of poor flowability. The following was the formula [21]:

$$\text{Hausner's ratio} = \frac{\text{Tapped bulk density}}{\text{Loose bulk density}} \quad \text{(Equation 4)}$$

E. Carr's Compressibility Index (CCI)

Carr's compressibility index was calculated with the

formula as follows [21]:

$$\%CCI = \frac{TBD-LBD}{TBD} x\ 100 \ldots\ldots\ldots\ldots \quad \text{(Equation 5)}$$

Where, TBD = Tapped bulk density, LBD= Loose bulk density.

F. Heckel plot

A single punch machine (KBR Manual Hydraulic Press) equipped with a round flat-faced stainless steel die cavity with a diameter of 10 cm was used for the preparation of the compacts. In advance of the compression, the punch faces and the die wall were lubricated with a 2% magnesium stearate suspension in acetone. Six tablets of 200 ± 3 mg weight were prepared at each of the 6 different compression pressures (0.5, 1, 1.5, 2, 2.5, and 3 tons). Compact weight, hardness, diameter, and thickness of out-of-die tablets, and radius, were measured. Compact density was calculated using its weight (w), diameter (d), and thickness (t). Out-of-die tablet relative density at any compression pressure was calculated as the ratio of compact density to true density. The negative natural logarithm of tablet porosity was taken as its densification. Finally, the graph of ln (1/1-Rd) versus pressure was plotted to analyze the compression properties of the powder compacts [21].

4.3.2 Scanning Electron Microscopy (SEM):

Morphological analysis was performed using Scanning Electron Microscopy (JEOL JSM-6360, Japan). The samples were fixed on a brass stub using double-sided adhesive tape and made electrically conductive by coating in a vacuum (6 Pa) with platinum (6 nm/min) using a Hitachi Ion Sputter (E-1030) for 120 s at 15 mA. The SEM images were analyzed using an image analysis system (ImageInside Ver 2.32) [32].

4.3.3 Particle size determination:

The particle size of all freshly prepared BM-SD formulations was determined by using Zetasizer version 11(Malvern Instruments, Worcestershire, UK) with the manufacturer's software [32].

4.3.4 Drug content determination:

The percentage of drug content in SD was estimated by dissolving the SD equal to 10 mg of BM in 100 mL of methyl alcohol. This solution was further diluted with phosphate buffer (pH 6.8) and the absorbance of each of these solutions was measured at 273 nm [32].

$$\text{Percentage drug content} = \frac{\text{Actual amount of drug}}{\text{Theoretical amount of drug}} 100 \quad \text{(Equation 6)}$$

4.3.5 Saturation solubility studies:

To the glass vials holding 5 mL of phosphate buffer (pH 6.8), each SD was introduced individually. These vials were shaken in the orbital shaker cum incubator at 20 rpm for 24 h at 37±0.5 °C. Samples were filtered using Whatman filter paper (No. 41), and relevant dilutions with phosphate buffer (pH 6.8) were prepared, and finally evaluated spectrophotometrically by detecting absorbance at 273 nm [33]. Depending on the results of drug content and saturation solubility studies, one composition was optimized and used to prepare tablets.

4.4 Formulation of tablet:

The optimized SD (SD5) was passed through # 80 followed by the addition of calculated amounts of each tableting ingredient o form a uniform blend. This blend was gradually supplemented with an appropriate amount of the granulating agent to support tablet formation. The resulting granules were passed through #22/44 and dried at 40 °C for 12 h. On completion of drying, binders HPMC K4M, and MCC were incorporated, followed by the addition of talc as glidant and magnesium stearate as lubricant. The tablets were prepared by the direct compression and evaluated for several pharmacopoeial tests to optimize the formulation [34].

4.5 Evaluation of tablets:

4.5.1 Weight variation:

All 20 tablets from the optimized tablet formulation batch were separately weighed individually. The weight of each tablet was compared against the average weight of

tablets. The following formula was then used to calculate the percent weight variation [35].

$$\%\text{Weight variation} = \frac{\text{Individual weight}}{\text{Average weight}} \times 100 \qquad \text{(Equation 7)}$$

4.5.2 Thickness:

To measure the thickness and diameter, a digital vernier caliper was used. This test was done to examine the uniformity in tablet size and thickness. It was determined by arbitrarily picking 5 tablets from each formulation and measuring with a vernier caliper to calculate the mean thickness [35].

4.5.3 Hardness:

The hardness of the tablet depends on the quantity of material filled in the die and the amount of compressional force applied. Tablet hardness affects the resistance offered by them during handling, transportation, or storage before use. Tablets' hardness was determined by employing a Monsanto hardness tester [36]. The top plunger was pushed against a spring by spinning the threaded bolt while the lower plunger was kept in contact with the tablet, and the reading recorded was zero. A hardness test for tablets was conducted on randomly selected 5 tablets from the optimized batch. This force applied continued until the tablet broke. The fracture force was measured and subtracted from the zero-force value [37].

4.5.4 Friability:

The Veego friabilator was used to test friability. Normally, a pre-weighed tablet sample from each created batch was placed in a friabilator and rotated 100 times. After a subsequent reweigh, the tablets were measured again, and the following formula was used to determine the % loss [35].

$$\%\text{Friability} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \qquad \text{(Equation 8)}$$

4.5.5 *In vitro* disintegration time:

The USP disintegration tester was used to conduct a disintegration test, (Model ED-2L, Electrolab, Mumbai). In order to perform this test, the disintegration medium used was phosphate buffer (pH 6.8) kept at 37 ±2 °C. A total of 6 tablets were put in the tubes of a tester, and the amount of time it took for each tablet to disintegrate and dissolve in the medium without leaving any residue was noted [37].

4.5.6 Drug content:

The weight of randomly selected 5 tablets was recorded individually and the average weight was calculated. These tablets were powdered in a mortar using a pestle. The powder weight equivalent to 40 mg of BM was transferred into a volumetric flask containing phosphate buffer (pH 6.8) and the volume was made up to 100 mL. Further, the solution was kept aside for a day to allow for the complete dissolution of the drug. This solution was then diluted to obtain a solution of strength 10 µg/mL. This diluted solution was filtered, and absorbance was recorded at 273 nm on a UV-visible spectrophotometer [36].

4.5.7 XRPD Analysis:

XRPD analysis of powdered samples was accomplished by using an X-ray diffractometer (Bruker, Germany). It was used to measure the angle of diffraction in the range of 4 - 40º within the reproducibility limit of ±0.001. The rate meter's scanning speed of 2ºθ/min was used to record the XRPD pattern automatically. The XRPD patterns of pure drug and formulation were recorded and analyzed [38].

4.5.8 FTIR Analysis:

A Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRS-FTIR) (Bruker, Germany) was used to obtain the FTIR spectra of BM and tablet formulation. Dry KBr was mixed with 2 to 3 mg of samples, and the spectra were scanned throughout a wave number in the range of 4000 to 400 cm^{-1} [39].

4.5.9 DSC Analysis:

The DSC thermogram of the BM and its tablet formulation was used obtained using Hitachi 7020 to assess its thermal behavior. The sample was heated at a

constant rate of 10 °C/min throughout a temperature range of 25 - 350 °C while being hermetically enclosed in aluminum pans. An inert atmosphere was maintained by purging with nitrogen at a flow rate of 60 mL/min [40].

4.5.10 Dissolution study:

In vitro drug release studies provide information about the amount of drug released from the formulation. The dissolution of the tablets was examined using USP XXII type II dissolution test equipment (Electrolab-TDT 08L). These paddles are placed in a vessel containing the dissolution medium in the dissolution test apparatus, rotating at 50 r.p.m. Aliquots were taken out at scheduled time intervals of up to 9 hrs and evaluated spectrophotometrically using a UV-visible spectrophotometer (Jasco V630) at a maximum wavelength of 273 nm [35].

4.5.11 Drug release kinetics study:

The drug release profile of the optimized SD was examined to calculate R2 and to verify the type of model it follows. Models tested include zero-order, first-order, Higuchi models, and Korsmeyer and Peppas model [41].

4.5.12 Stability Study:

Investigation of physical changes in an optimized tablet formulation was performed by short-term stability studies such as drug content and drug release. As a formal prerequisite for the licensing of pharmaceuticals for human consumption, the FDA and ICH define standards for stability testing of new medicinal products. To conduct stability tests on promising formulations, tablets were kept at a temperature of 40 °C and relative humidity (75%) for three months [42,43].

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تكوين وتطوير وتقييم أقراص تشتت صلبة بالتجفيف بالرش لبوسنتان مونوهيدرات لتحسين ملف الذوبانية

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ملخص

بوسنتان مونوهيدرات (BM) يستخدم لعلاج ضغط الشرايين الرئوية. لديه قابلية ضعيفة للذوبان في الماء وانتشارية حيوية منخفضة. تهدف الدراسة الحالية إلى تحسين معدل الذوبان للأدوية باستخدام إيودراجيت® إي بي أو عن طريق التجفيف بالرش. تم استخدام الدواء وإيودراجيت® إي بي أو بنسبة 1:1، 1:2، 1:3، 1:4، و 1:5 (وزن/وزن) لإنتاج التراكيب SD1 إلى .SD5 أظهر SD5 بنسبة الدواء إلى الحامل 1:5 زيادة ملحوظة إحصائيًا في ذوبانية التشبع ومحتوى الدواء. تم إنشاء 6 تركيبات للأقراص تحتوي على SD5 ومكونات التحبيب، مرقمة من F1 إلى F6 وتم معالجتها. لوحظت أعلى معدل ذوبان وإطلاق للدواء في التركيبة F2 التي تحتوي على HPMC K4M 26.36% و MCC. 23.63% الآلية المحتملة المعنية بذوبان BM في SD هي شكله البلوري والتأثير المحلل للدواء الذي يتمثل في التآزر بين BM وإيودراجيت® إي بي أو. قد يكون قوة الشد العالية وقلة التلف ووقت الانحلال الطويل نتيجة لتأثير الربط المظهري للحامل. قد تكون الخلط المباشر لـ SD مع HPMC قد ساهم في تحسين التجانس لـ SD داخل مصفوفة الأقراص وملف الإفراج. هذه الدراسة أوضحت ملائمة التجفيف بالرش في تحضير SD من BM مع إيودراجيت® إي بي أو، مما يمكن أن يحسن قابلية الذوبان والاستقرار للدواء.

الكلمات الدالة: بوسنتان مونوهيدرات، إيودراجيت® إي بي أو، قابلية الذوبان، الذوبان، التشتت الصلب، التجفيف بالرش.

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Chemical Composition of the Essential Oils of the Flowers *Asphodelus aestivus Brot.* Grown Wild in Jordan

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ABSTRACT

Recent technological developments and methodological advances in GC-MS have become a common tool for investigating the quantity, quality, and chemical diversity of plant secondary metabolites. The flower parts of Asphodelus aestivus Brot. were studied, leading to the isolation and identification of various secondary metabolites, primarily essential oils: alcohol (26.9%); aldehyde (23%); alkanes, acetate derivatives, and aliphatic derivatives (19.2%); ketones (7.7%); and epoxides (3.8%). The principal oil components were 18.79% vetocitral C (trans), 17.27% hexadecyl acetate, 14.5% hexanal (2E), and 9.6% sabinene hydrate (trans). The identification of oil components was performed by matching their spectra with the mass spectra data bank.

Keywords: Medicinal plants, *Asphodelus aestivus* Brot, Jordan flora.

INTRODUCTION

Asphodelus L. is a genus comprising about 20 species in the Asphodelaceae family. They are native to Europe, North Africa, and Asia, primarily the Mediterranean. Many have a small rhizomatous crown and thick, fleshy roots that were first described by Carl Linnaeus in 1753 [1]. *Asphodelus aestivus* Brot, syn. (*Asphodelus microcarpus* Salzm. et Vivi) is a stout, robust herb with roots consisting of several spindle-shaped tubers, widely distributed across the coastal Mediterranean region [2].

Medicinal plants and their respective phytochemicals, mainly secondary metabolites, are utilized to address specific nutrient deficiencies and sustain secure food and primary healthcare medicines [3]. Natural materials derived from plants have played a significant role in drug discovery and in improving the healthcare system [4-8]. The World Health Organization (WHO) estimates that approximately 80 percent of the world's population relies on natural resources for their basic healthcare needs, while the remaining 20 percent uses integrated natural resources [8]. In the 21st century, 11 percent of the 252 essential medicines considered by the WHO as crucial came exclusively from flowering plants [9]. The species *Asphodelus* L. (Asphodelaceae) is consumed in large quantities in the cuisines (e.g., soups, pastries, etc.) of several countries and cultures [4]. *Asphodelus aestivus* is found on agricultural lands, around roads, and on calcareous soils in pastures in the Mediterranean basin [3, 7]. *Asphodelus aestivus* is used for food and as a folk medicine for eczema, stomach diseases, and hemorrhoids [6, 7]. Currently, there is a growing interest in phytochemicals as potential new sources of natural antioxidants, with the goal of using them in foods and pharmaceutical preparations to replace synthetic antioxidants [10].

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EXPERIMENTAL

Plant Material

The flowers of *A. aestivus* were collected from the Rujm Al-Shoof area (15 km northeast of Amman) in February 2022. The taxonomic identities of the collected plant material were confirmed with the assistance of a plant taxonomist (Dr. Mohammad Gharaibeh, Faculty of Agriculture, Jordan University of Science and Technology) and by comparing a collected voucher specimen with those of known identity in the herbarium of the Faculty of Agriculture, Jordan University of Science and Technology. A voucher specimen (ID No.: Phar 09-4) of the collected plant was deposited in the research laboratory of the Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy, Jordan University of Science and Technology.

Oil distillation

The flowers of the collected plant material of *A. aestivus* were air-dried and ground to about a 0.5 mm particle size (30-35 mesh). Essential oils were obtained by subjecting 395 g of the ground materials to hydro distillation using the Clevenger-type apparatus (JSGW, India) for 4 h. The obtained oils (n = 2) were dried over anhydrous sodium sulfate, Na2SO4, and stored in dry, dark glass bottles at 4°C for later analysis.

Analysis of the Essential Oils

A quantitative analysis using gas chromatography with a flame ionization detector (GC-FID) was conducted using a Hewlett Packard HP-8590 gas chromatograph equipped with a split-splitless injector (split ratio 1:50) and an FID detector. The column (OPTIMA5 (5% diphenyl 95% dimethyl polysiloxane)) was a fused silica capillary column (30 m x 0.25 mm; 0.25 μm film thickness). The oils were separated under a linear temperature program set at a 3°C/min heating rate from 60-250°C and then held at 250°C for 5 min. The temperature of the injector and detector were maintained at 250°C and 300°C, respectively. The relative peak area for each component of the oil was measured. The concentrations of the oil components were calculated as a percentage content using their relative peak areas assuming a unity response by all components. Each sample was analyzed twice.

GC-MS Analysis

A GC-MS analysis was conducted on a Varian Chrompack CP-3800 GC/MS/MS-200, equipped with a split-splitless injector and a DB-5 GC column (5% diphenyl 95% dimethyl polysiloxane, 30 m x 0.25 mm ID, 0.25 μm film thickness). The injector temperature was set at 250°C with a split ratio of 1:10. Detector and transfer-line temperatures were 160°C and 230°C, respectively. A linear temperature program was used to separate the different oil components. Temperature programming was applied at a 3°C/min heating rate, starting from 60°C to 250°C, and then held at 250°C for 5 min. The mass detector was set to scan ions between 40-400 m/z using full scan mode and electron impact (EI, 70 eV). Each sample was analyzed twice. A hydrocarbon mixture of n-alkanes (C8-C20) was separately applied on a GC-MS using the same chromatographic conditions. The Linear retention index (arithmetic Kovat's index) was calculated for each component separated by GC-MS using the values of its retention time and the retention times of the reference n-alkanes applying the Van Den Dool equation [11-14 ,16,17].

The identification of oil components was performed by matching their spectra with the mass spectra data bank (Wiley, Nist, and Adams 2007 libraries), and also by comparing their calculated arithmetic indices with the reported values in the literature [11, 13, 15 -17].

RESULTS AND DISCUSSION

The simultaneous use of mass spectral and retention (Kovat's) index matching enabled the unequivocal identification of more than 98% of the components in the collected oil obtained from the flowers of the plant under study, as determined by GC and GC-MS. The oil yield (expressed as % v/w of dried material) was 0.35%. The analyses permitted the identification of 26 compounds in

the oils of A. aestivus. The identified components and their corresponding contents are presented in Table 1: Octane (1), hexanal (2), hexanal (2E) (3), N-heptanal (4), acetonyl acetone (5), ethyl hexanoate (6), octanol (N-) (7), sabinene hydrate (trans) (8), vetrocitral C (trans) (9), isopulegol (neoiso-) (10), terpinol (gamma-) (11), octenol acetate (2E) (12), geraniol (13), undecanal (14), decadienol (2E,4E) (15), docdecanol (n-) (16), pentadecane (17), liguloxide (18), hepatadecane (n-) (19), heptadecane (n-) (20), longifolol (21), acoron (22), farnesyl acetate (2E, 6E) (23), nonadecane (n-) (24), methyl hexadecanoate (25), and hexadecyl acetate (26).

The essential oil was characterized by high percentage levels of aldehydic volatile oil (hexanal, hexanal (2E), N-heptanal, vetrocitral C (trans), undecanal) (40.94%); followed by alkane and acetate derivatives (octane, ethyl hexanoate, pentadecane, hexadecane (n-), heptadecane (n-), farnecylacetate (2E,6E), nanodecane (n-), methylhexadecanoate, hexadecyl acetate) (31.15%); alcoholic volatile oil (octanol (N-) isopulegol (neoiso-), terpinol (gamma-), octenol acetate (2E), geraniol, decadienol (2E,4E), dodecanol (n-), longifolol)) (14.38%); bicyclic monoterpene (sabinene hydrate (trans) (9.6%); ketanes volatile oil (acetonyl acetone, acorone) (2.9%) and epoxides (liguloxide) (1.68%).

Vetrocitral C (trans) 18.79% was the principal oil component, with 17.27% hexadecyl acetate, 14.5% hexanal (2E), and 9.6% sabinene hydrate (trans) (shown in bold in Table 1) as the major oil constituents (8).

The identification of oil components was performed by matching their spectra with the data bank mass spectra (Wiley, Nist, and Adams 2007 libraries).

Table1: Chemical composition the essential oil hydro-distilled from the flowers parts of Jordanian *A. aestivus*

No	RI exp	RI lit	Content %	Compound
1	801	800	2.75	Octane
2	802	801	2.15	Hexanal
3	851	855	14.5	**Hexanal (2E)**
4	903	902	3.5	N- Heptanal
5	925	924	1.3	Acetonyl acetone
6	998	998	1.7	Ethyl hexanoate
7	1070	1068	3.6	Octanol (N-)
8	1100	1098	**9.6**	**Sabinene hydrate (trans)**
9	1107	1105	18.79	**Vetrocitral C (trans)**
10	1171	1171	1.3	Isopulegol (neoiso-)
11	1199	1199	2.2	Terpinol (gamma-)
12	1206	1209	1.1	Octenol acetate (2E).
13	1252	1249	1.9	Geraniol
14	1308	1306	2.0	Undecanal
15	1321	1321	1.1	Decadienol (2E,4E)
16	1469	1470	1.5	Dodecanol (n-)
17	1498	1500	1.3	Pentadecane
18	1537	1536	1.68	Liguloxide
19	1598	1600	1.17	Hexadecane (n-)

No	RI exp	RI lit	Content %	Compound
20	1700	1700	1.56	Heptadecane (n-)
21	1715	1714	1.68	Longifolol
22	1819	1820	1.6	Acorone
23	1844	1846	1.5	Farnesyl acetate (2E, 6E)
24	1897	1900	2.8	Nonadecane (n-)
25	1981	1921	1.1	Methyl hexadecanoate
26	2002	2003	17.27	**Hexadecyl acetate**
			40.94	Aldehide v. oils 31.15 Alkane and acetate derivatives v. oils 14.38 Alcohol v. oils
			9.6	Bicyclic monoterpene v. oils
			2.9	Ketanes v. oils
			1.68	Epoxides v. oils

RI exp: Linear (arithmetic) retention index calculated on DB-5 equivalent column
RI lit: reference retention index value from literature
*Average% content of 4 determinations (2 oil samples, 2 replicates each), for which the standard deviation (SD) values were within 2% (+2%) of the mean
Compounds in bold are the major components (≥1.0%)

CONCLUSION

The flower parts of Asphodelus aestivus Brot. were studied, leading to the isolation and identification of various secondary metabolites, mainly essential oil. The major essential oil components were vetrocitral C (trans), hexadecyl acetate, hexanal (2E), and sabinene hydrate (trans). The identification of oil components was performed using GC-MS.

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Conflicts of interest:

The authors have declared that there is no conflict of interest associated with the publication.

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الزيوت الأساسية للزهور نبتة أسفوديلاس *ايستيفاس* .(*Asphodelus aestivus* Brot) والتي تنمو بريا في الأردن

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ملخص

أصبحت التطورات التكنولوجية الحديثة والتطورات المنهجية في كروماتوغرافيا الغاز ومقياس الكتلة أداة شائعة لدراسة كمية ونوعية والتنوع الكيميائي للمستقلبات الثانوية النباتية. تمت دراسة أزهار نبتة أسفوديلاس ايستيفاس، والتي تنمو بريا في الأردن، مما أدى إلى عزل وتحديد مختلف مستقلبات ثانوية مختلفة، ولا سيما الزيوت الأساسية: الكحول (26.9 %) ؛ الألدهيد (23 %)؛ والألكانات ومشتقات الأسيتات والمشتقات الأليفاتية (19.2 %) ؛ الكيتانات (7.7%) والأكاسيد الخارجية (3.8%). كان المكون النفطي الرئيسي 18.79٪ فيتروسيترال (Cترانس)، 17.27٪ أسيتات سداسي أسيل، 14.5٪ سداسي (2E)، 9.6٪ هيدرات سابينين (ترانس). تم تحديد مكونات الزيت عن طريق مطابقة أطيافها مع أطياف كتلة بنك البيانات.

الكلمات الدالة: النباتات الطبية، غوصلان، نباتات الاردن.

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Extracellular Synthesis of Magnesium Oxide at Nano and Bulk Scale: Antifungal Effect Against *Candida albicans, Aspergillus niger*

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ABSTRACT

The antifungal activity of magnesium oxide nanoparticles (MgO-NPs) prepared by the cactus plant (Opuntia ficus indica) at the nano and macro scale was evaluated against important human pathogens: Candida albicans and Aspergillus niger. The nanoparticles were characterized using UV–Vis, FTIR, DLS, EDX, and FESEM. UV–Vis analysis revealed a peak at 300 nm, and FT-IR analysis showed that the biomolecules played an important role in ions reduction, leading to the growth of MgO-NPs. A peak close to 400 cm−1 was observed, indicating Mg-O-Mg bonding. EDX analysis confirmed the presence of MgO-NPs. MgO-NPs were identified as nanospheres with diameters between 15.5 and 78.01 nm (average 42.28 nm), while MgO-Bulk was identified as macrospheres with lengths between 105.2 and 1313.9 nm (average 356.09 nm) using FESEM. Z-average sizes by DLS analysis were 46.04 nm and 377 nm. In vitro antifungal assays were evaluated using two methods: well diffusion and the microdilution method, and MgO-NPs showed the highest effect in both. The Minimum Inhibitory Concentration (MIC) for MgO-NPs was equal to 1.5 mg/mL and 6.25 mg/mL for C. albicans and A. niger, respectively.

Keywords: MgO nanoparticles; Biosynthesis; *ficus indica*; Antifungal activity; *Candida albicans; Aspergillus niger*.

1. INTRODUCTION:

From ancient times, fungal infections have significantly contributed to the ever-increasing morbidity and mortality rate[1]. The increasing clinical and microbiologic resistance of Candida spp. and Aspergillus spp. isolates to several antifungal agents is becoming a serious problem[2,3]. Death rates due to invasive fungal infections are often 50% or higher[4]. The risk of failure of surgical operations and medical treatment will increase in the absence of effective drugs[5]. Nanotechnology is a science that uses various techniques to synthesize nanoparticles[6]. Nanoparticles possess several properties compared to larger particles of the same material, mainly due to their large surface area in relation to volume. The influence of this factor is significant, as materials larger than 100 nm are expected to have physical and chemical properties regardless of size, but this is different for nanoparticles. Foremost among them is their antifungal properties[7,8]. Metal oxides were attractive because of their easy modifiability and a variety of shapes[9]. Among metallic nanoparticles, extensive research has been carried out using MgO-NPs and their applications in the biomedical field as drug delivery system[10], anti-microbial[11], and anti-cancer agents[12], water purification and also in pharmaceutical products[6].

MgO has gained keen interest due to its ecofriendly nature, the availability of greater specific area, biocompatibility, low cost, and the easy convenience of its sources[6]. In addition, The Food and Drug Administration (FDA) recommended MgO-NPs as safe materials[13]. Studies in this regard are few compared to other minerals. Presently, green synthesis of metal nanoparticles using plant extract gets a lot of attention since it is eco-friendly

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and cost-effective [9]. It has been known since the early 20th century[9]. Cactus is one of the plants used as bioactivators for the fabrication of nanoparticles, which has many types. In recent years, the cacti genus has received a lot of attention for its therapeutic benefits, in the foreground, its antimicrobial effect[14]. MgO-NPs synthesis via green route is limited in the literature and few studies on MgO-NPs green synthesis using *Nephelium lappaceum L.*[15], *Azadirachta indica*[16], *Aloe vera* [17], *Betel leaf* [18], *Sargassum wighti* [19], *Rhizophora lamarckii*, were reported previously. In this study, *Opunita ficus indica* extract mediated green synthesis of MgO-NPs, which was investigated for the first time. *O.F.I* is a well-known elaborate plant that belongs to *Cactaceae*[20]. It has been used in traditional folk medicine for its antifungal and antibacterial effects [21].

2. MATERIALS AND METHODS

1.2 Materials:

The Opuntia ficus indica (O.F.I) cladodes were freshly collected from wild plants growing in Homs, Syria (34°21′N 38°19′E). Double-distilled water (DDW) was used in the experiments. Sodium hydroxide 98% (Medex, UK), Ethanol GR (Eurolab, UK). All the chemical materials were obtained from Aldrich. Fungal species: Aspergillus niger was obtained from the Microbiology lab at Pharmacy College, Al-Baath University, Syria, Candida albicans ATCC 10231 was obtained from the Atomic Energy Commission, Damascus, Syria.

Equipment: Ultrasonic bath (POWERSONIC 405, Hwashin Technology Co., Korea), Sensitive balance (Sartorius TE214, Germany), UV-1800 spectrophotometer (Shimadzu, Japan), Rotary evaporator (Heidolph Instruments, Germany), Ultrapure TM water purification system (Lotun Co., Ltd., Taipei, Taiwan), Zetasizer instrument (DLS; Zeta-size Nano-ZS; Malvern Instruments, UK), FTIR spectrometer (SHIMADZU), mixer (Germany DI 18 basic), FESEM (European).

2.2 Methods: Experiments were performed triples.

1.2.2 Preparation of the extract: The healthy Opuntia ficus indica was collected, washed under running tap water, dried for 5 minutes at room temperature, and finely chopped. Briefly, 30 g of the plant was suspended in a 500ml beaker (100:50 mL) (DDW: Ethanol) and treated with ultrasound for 30 min at 50°C. The obtained extract was filtered through Whatman number 1 filter paper, and it was concentrated by a rotary evaporator until it removed all the ethanol. A freshly prepared extract was used for the synthesis of MgO-NPs.

2.2.2 Biosynthesis of MgO-NPs: In the experiment, MgO-NPs were prepared by slowly mixing 10 ml of plant extract with 90 ml of 10^(-3) M aqueous solution of magnesium nitrate dropwise under vigorous agitation by a mixer and adding NaOH (0.1 N) until pH reached 11. Then, the mixture was left overnight by magnetic stirring and at 35°C. The magnesium nitrate ions were reduced to magnesium oxide nanoparticles using the plant extract. The formation of MgO-NPs has been observed by a color change. Thereafter, the solvent of nanoparticles was evaporated by a rotary evaporator. Finally, the obtained precipitate was dried at 60°C and calcinated at 500°C for 3 hours to produce MgO-NPs.

3.2.2 Characterization of MgO-NPs:

the formation of MgO-NPS was confirmed by:

- **Ultraviolet-Visible (UV–Vis) Spectroscopy**

The optical features of MgO-NPs were determined using UV–Vis spectroscopy. The highest absorbance (λmax) of a stock solution of the MgO-NPs, diluted with distilled water (1:1 ratio), was measured in the wavelength range from 200 to 600 nm to confirm the presence of the specific Surface Plasmon Resonance (SPR) peak of MgO-NPs.

- **Fourier Transform Infrared Spectroscopy (FTIR) Analysis**

The MgO-NPs, which were calcinated at 500°C, and the dried form of the extract at 80°C were pelletized with KBr and analyzed using an FTIR spectrometer[22]. The absorption was recorded in the field extending from 400 to 4000cm^{-1}.

- **Dynamic Light Scattering (DLS)**

DLS was used to measure the mean diameter and polydispersity index (PDI). It was performed by the Zetasizer instrument at 25°C. The particle diameter determined in this way is known as the hydrodynamic diameter. The value of PDI ranges between 0 and 1, with a value close to 0 being mono-dispersed and a value close to 1 representing a poly-dispersed sample. It is important to synthesize nanoparticles with a lower size and higher absorbance[23].

- **Field Emission Scanning Electron Microscope (FE-SEM) Analysis**

Samples were prepared by placing a drop of colloidal on a slide and drying it at 25°C. FE-SEM images were taken, and Image J and Origin 2017 were utilized to prepare histograms for dimensions.

- **Energy-dispersive X-ray spectroscopy (EDX)**:

EDX analysis confirmed the presence of MgO -NPs. EDX relies on the characteristic X-ray emission of MgO.

***4.2.2 In Vitro* Antifungal Activity of MgO-NPS**

The antifungal activity of the MgO-NPs was evaluated using agar well diffusion against Aspergillus niger and Candida albicans ATCC 10231. The tested fungal strains were grown on Sabouraud Dextrose agar plates and incubated for 1, 5 days at 37°C and 25°C for C. albicans and A. niger, respectively[2] [24]. The turbidity of the suspension: for *Candida Albicans*, the turbidity was adjusted by spectrophotometry to an optical density of 0.08-0.1[2], and for *Aspergillus niger* was an optical density of 0.09 to 0.13[24]. One milliliter was uniformly distributed on Czapek dox agar plates. Using a sterile cork-borer, wells (8 mm) were cut; The wells were closed with a drop of agar so that the nanoparticles would not settle to the bottom of the well, 50,100 µL of MgO-NPs and 50,100 µL of MgO-Bulk were transferred to each well individually and left for 2 h at 25 °C, and then the plates were incubated for 1, 5 days at 25, 35°C for *C. Albicans, A. niger* respectively. After incubation, the inhibition zones are determined and recorded. Moreover, different concentrations of MgO-NPs were evaluated as antifungal to detect the minimum inhibitory concentration (MIC).

5.2.2 NCCLS Microdilution Method

This method was used as described in the National Committee for Clinical Laboratory Standards. The MIC of MgO-NPs was defined as the lowest concentration which resulted in a prominent decrease in turbidity compared to that of growth-control wells[25].

3. RESULTS AND DISCUSSION

1.3 UV–visible spectroscopy:

MgO ions were bioreduced to MgO-NPs when Opuntia ficus-indica extracts were added. The formation of MgO-NPs was confirmed by UV-Vis spectrophotometer studies within a range of 200-600 nm. The specific SPR peak of MgO-NPs after 24 hours was found to be centered at 300 nm in the spectrum, indicating the presence of MgO-NPs. This finding aligns with studies by Dobrucka R[26], and Prasanth R *et al* [27]**.** The obtained SPR peak confirms the reduction of Mg (NO3)2 to MgO-NPs, and it is evident that the phytochemicals present in O.F.I may function as a capping and stabilizing agent toward the MgO-NPs. MgO-Bulk showed a modest peak at 300 nm, lower than the nanoparticle peak. As shown in Figure 1, the frequency and width of the surface plasmon absorption depend on the size and shape of the metal nanoparticle[28].

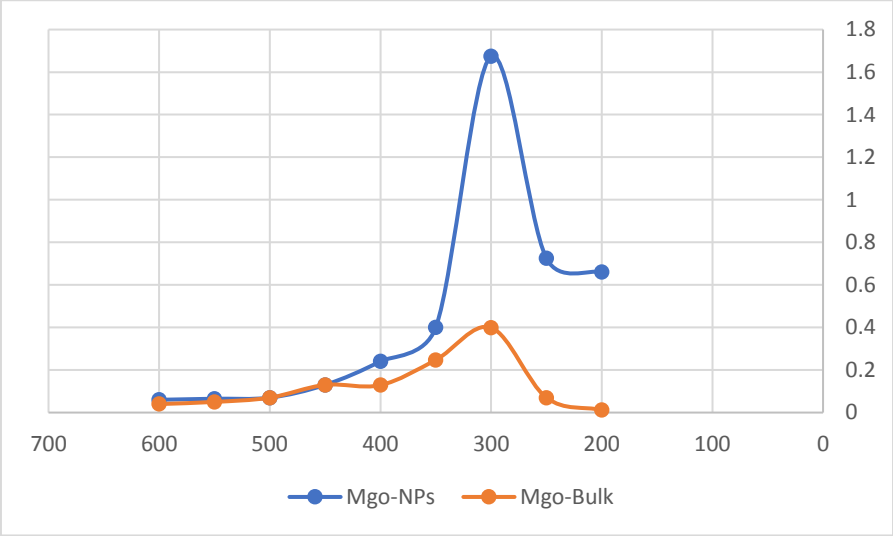
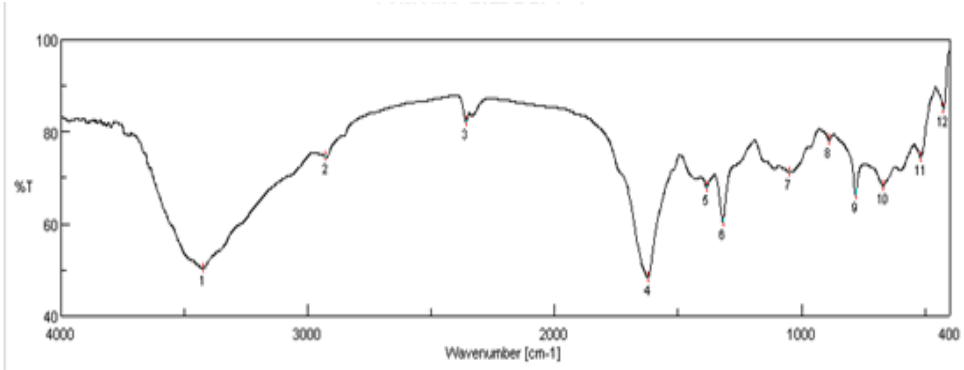


Fig 1. UV-vis absorption spectrum of MgO-NPs, MgO-Bulk

2.3 Fourier transform infrared spectroscopy (FTIR) analysis: FT-IR measurements were conducted to identify the possible biomolecules responsible for the reduction of Mg^(+2) and capping of the MgO-NPs synthesized using O.F.I extract. From the two spectra (Figure 2a, b), we observed a decrease in the quantities of plant components (alcoholic, amino compounds, alkanes, alkenes, carboxylic acids, etc.)[29]. This decrease is indicative of their participation in the fabrication of nanoparticles, as evident in the peak that expresses phenols and alcohols, which decreases in the spectrum of nanoparticles. There is also the appearance of a clear peak (7) in the nanoscale sample at 466 cm^(-1) (Fig. 2b), which corresponds to Mg-O [30,17]. This result is similar to an Indian study that showed a peak at 450-560 cm $^{-1}$[31 31], and in contrast to the result in Poland in 2016, they used the *Artemisia abrotanum* plant, which gave a peak at 407-419 cm $^{-1}$[26].

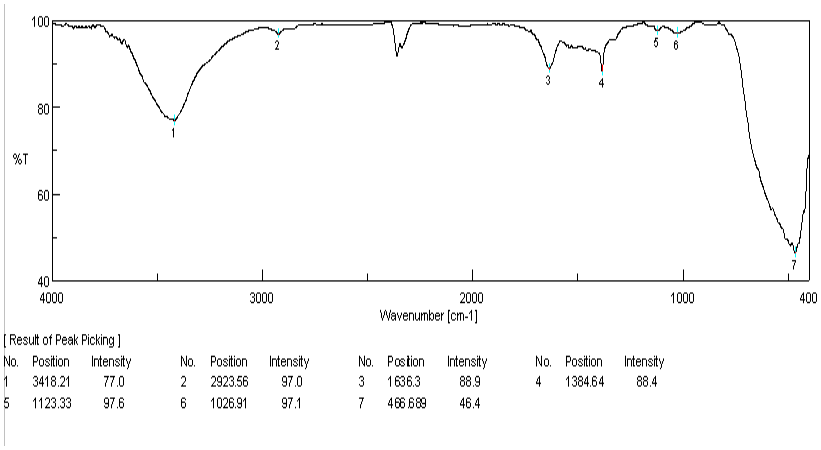
(a)



[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3424.96	50.3	2	2926.45	74.6	3	2358.52	82.2	4	1621.84	48.2
5	1383.68	68.0	6	1318.11	60.5	7	1049.09	71.2	8	888.059	78.2
9	780.065	66.4	10	670.142	68.2	11	517.793	74.4	12	424.263	85.1

(b)



[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3418.21	77.0	2	2923.56	97.0	3	1636.3	88.9	4	1384.64	88.4
5	1123.33	97.6	6	1026.91	97.1	7	466.689	46.4			

Fig 2. FTIR spectrum of (a) *Opunita ficus indica*, (b) MgO-NPs synthesis by *Opunita ficus indica*

3.3 Dynamic Light Scattering (DLS): DLS analysis of MgO-NPs synthesized using *O.F.I* and MgO-Bulk is shown in Fig. 3, with a Z-average size of 46.90nm. This differs from a study conducted in 2020 in which the dimensions of nickel oxide fabricated by *O.F.I* was 20-35nm[32], and this is due to the difference in the metal's ability to ionize[33], and it showed Z-average size at 377.3 nm for MgO-Bulk.

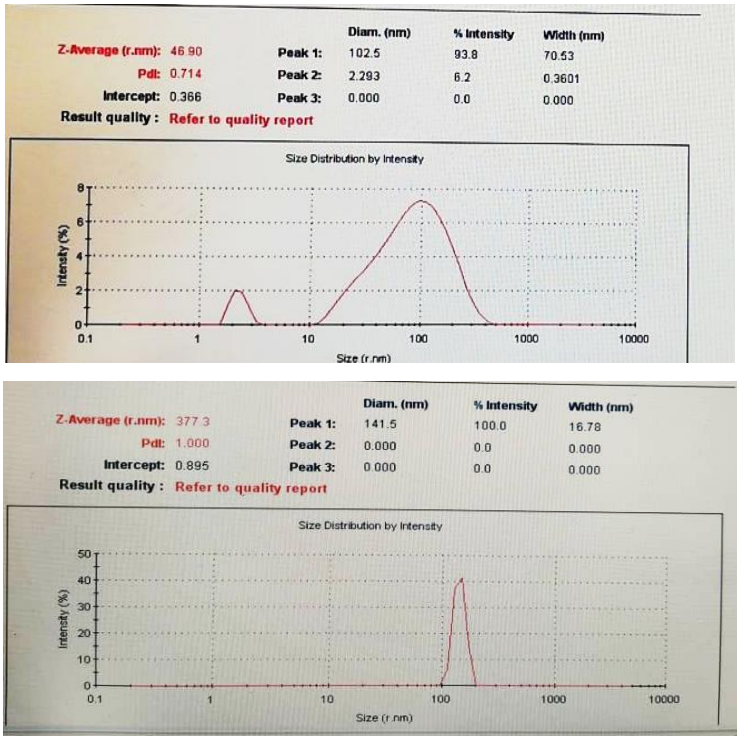
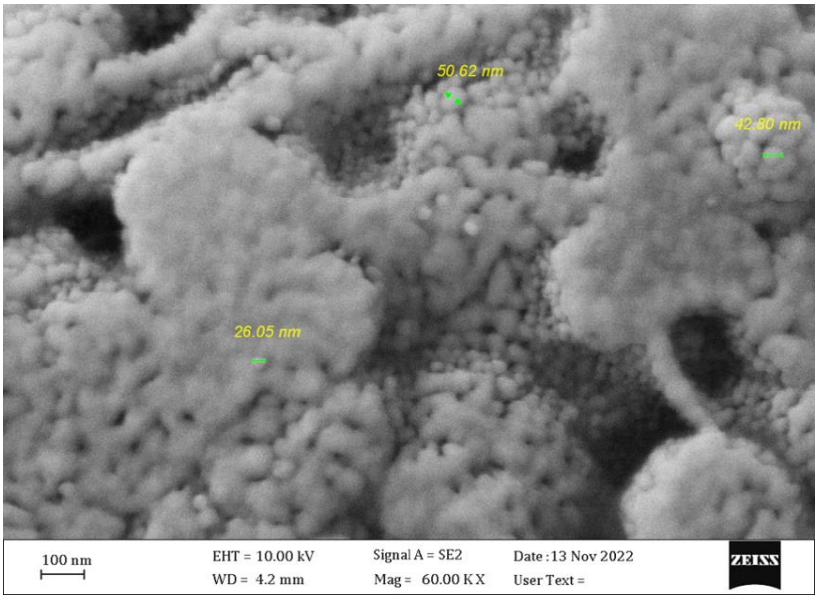


Fig 3. DLS of MgO-NPS, MgO-Bulk

4.3 Field Emission Scanning Electron Microscopy (FESEM):

FE-SEM analysis of biosynthesized MgO nanoparticles indicated a spherical shape with agglomeration and crystalline solid at both size nano and bulk particles. The size of particles which were synthesized by the green method was (42.28 as average) (Fig. 4), and MgO-Bulk was (356.04nm as average) (Fig. 5), due to histogram, and this is similar to the previously described. With DLS, this confirms the ability of plant metabolites to return salt ions.

(a)



(b)

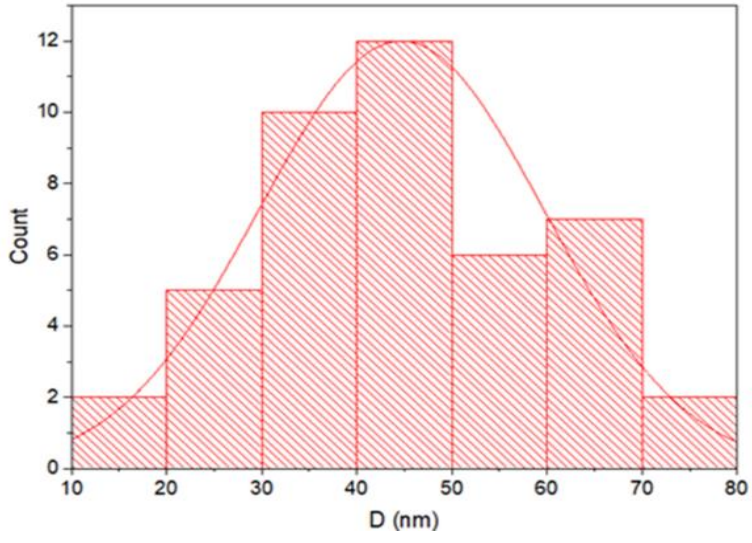
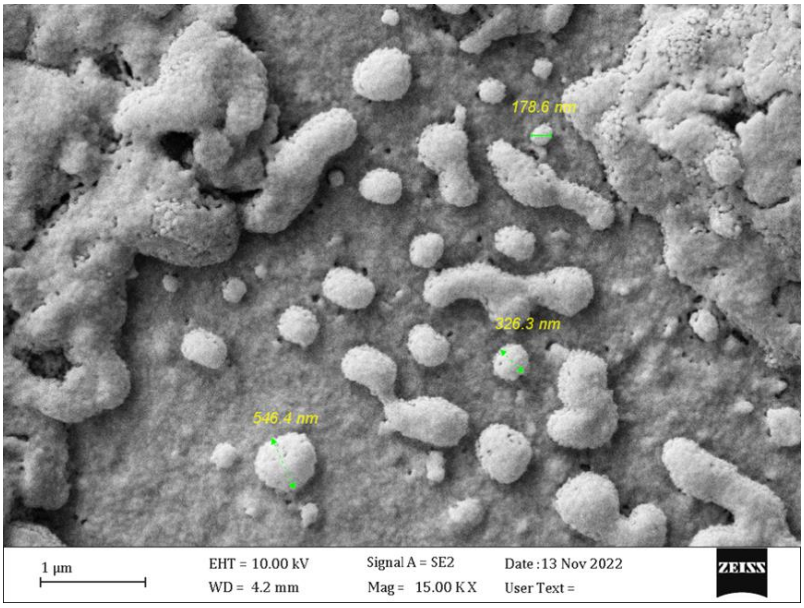


Fig 4. a: FESEM for MgO-NPs, b: Histogram of diameters

(a)



(b)

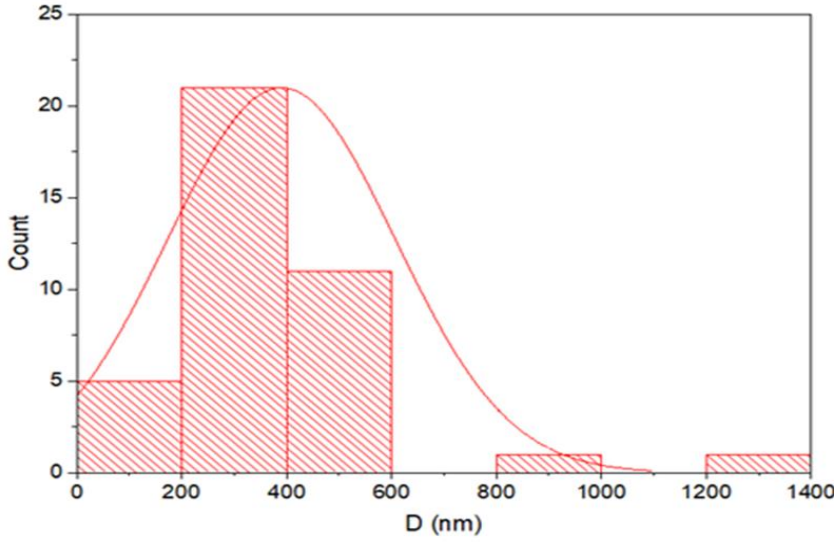


Fig 5. a: FESEM for MgO-Bulk, b: Histogram of diameters

5.3 Energy-dispersive X-ray spectroscopy (EDX):

The EDX analysis showed good signals for Mg,O together with remarkably stronger peaks, confirming that the pellets were MgO-NPs. The other elements are thought to have originated from the plant extract which is depicted in Fig. 6, thus confirming the formation of MgO-NPs.

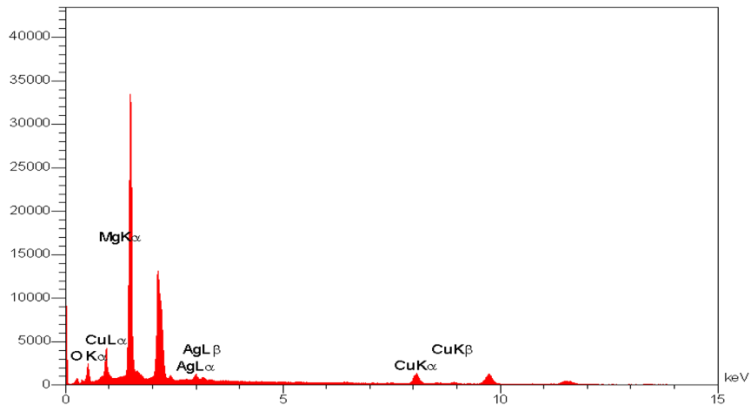


Fig 6. EDX analysis of MgO-NPs fabricated by green synthesis method

2.3 Antifungal effect:

1.2.3 well diffusion methods:

After 1 and 5 days of incubating the dishes in the microbiological incubator, the antifungal activity of the 100 and 50 mg/mL MgO-NPs solution was evaluated. The antifungal activity of the MgO-NPs solution was compared with MgO-Bulk against C. albicans and A. niger. The nanoparticles showed clear inhibition zones with diameters of 30 ± 0.5 and 25 ± 0.5 mm, respectively, whereas the bulk particles had no effect at both concentrations against C. albicans. The antifungal mechanism of MgO-NPs is not clear, unlike bacteria whose mechanism of action mainly depends on oxygen free radicals (ROS). For C. albicans, the mechanism may be due to the destruction of the integrity of the fungal cell membrane 34. It can be concluded that the effectiveness is directly related to the concentration used and increases with its increase, similar to the study of J. Chen et al [35]. In our results, the diameters of the inhibition zones were similar to the study of S. Vijayakumar et al [36] but in contrast to the study of E. Vidhya et al, which reported diameters of 21 ± 1.70 mm[37]. This difference may be due to the variation in the number of particles used. The study by Vidhya et al used 20 µL of nanoparticle suspension, while in our study, 100 µL was used, which directly affects the effectiveness[27].

Regarding *A.niger,* MgO-Bulk gave a small zone inhibition 17±0.5, 15±0.5 mm compared with MgO-NPs which was 25±0.5, 20±0.5 nm. This is similar to the study Vijayakumar S *et al* [36] where the inhibition zone was 24 ± 0.72 mm, and in contrast to the study by Vidhya E *et al* [38] which reported an inhibition zone of 16 mm. This may explain the difference in the pattern of the studied fungi. The different antifungal effect between the nano and bulk particles may be explained by the higher concentration of reactive oxygen species (ROS) in nanoparticles compared to bulk particles, causing oxidative stress on fungal cells, disrupting membrane structure, and altering fungal permeability, which may damage the cell and exhibit antifungal activity[39].

We infer that, due to the different sizes of the nanoparticles, it influences the antifungal effect[5]. Many reports indicate that size is the most important factor in the effectiveness of antibiotics, as size is a critical factor in the destruction of bacterial and fungal systems for many reasons. Small sizes allow accumulation and penetration of bacterial cells, causing damage and, thus, death of bacteria5. Metal oxide nanoparticles larger than 10 nm in size enhance permeability upon contact with the organism, and this is due to the surface area-to-volume effect that affects the specific surface. For this reason, they can also have an effect on direct toxicity mechanisms against bacteria and, consequently, subsequent death[5].

2.2.3 Microdilution methods:

Using broth microdilution, all species demonstrated sensitivity to MgO-NPs, as shown in Table 1. The MIC for MgO-NPs was 1.5 and 6.25 mg/mL against C. albicans and A. niger, respectively. This is an effective value as observed in in-vitro studies, and higher MIC levels were noted for MgO-Bulk against A. niger at 25 mg/mL. This finding aligns with the study by Nguyen NY[40]. The different antifungal effects between nano and bulk particles may be explained by the higher concentration of reactive oxygen species (ROS) in nanoparticles compared to bulk particles. This higher concentration causes oxidative stress on fungal cells, disrupts membrane structure, and alters fungal permeability, potentially damaging the cells and exhibiting antifungal activity[39].

Table .1: Minimum inhibitory concentration of MgO-NPs, MgOBulk against *c. albicans, A. niger* (mg/mL)

DMSO	MgO-Bulk	MgO-NPS	MIC
-	25	6.25	***Aspergillus niger***
-	-	1.56	***Candida albicans***

* Values were expressed as the means of Triples replicates.

4. CONCLUSIONS

Nanomaterials represent an emerging field that enables the more efficient use of antimicrobial compounds. In this study, the antifungal activity of MgO-NPs fabricated using Opuntia ficus indica extract was evaluated. The synthesized MgO-NPs underwent characterization through UV–Vis spectroscopy, FTIR, DLS, EDX, and FESEM analyses. The SPR peak at 300 nm observed during nanoparticle characterization confirmed the formation of MgO-NPs. FTIR analysis identified the functional groups responsible for the bioreduction and stabilization of the synthesized MgO-NPs using the extract. The dimensions of nano and bulk particles were measured through DLS and FE-SEM analysis, revealing spherical shapes, and the EDX spectrum confirmed the presence of magnesium (Mg) and oxygen. Furthermore, MgO NPs exhibited significant antifungal activity against Aspergillus niger and Candida albicans ATCC 10231, assessed through both Well Diffusion and Microdilution Methods, and were compared with bulk particles.

We believe that MgO-NPs hold great potential for applications in the pharmaceutical industry, serving as an alternative to antifungal agents facing resistance. MgO nanoparticles, whether used alone or in combination with other antifungal compounds, could present a superior choice for various future applications, such as coating medical devices or preserving food. Additionally, their cost-effectiveness makes them a favorable option for patients.

Conflicts of Interest: The authors have declared that there are no competing interests.

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الاصْطنَاع الخارج خلوي لجسيمات أوكسيد المغنيزيوم في المستوى النّانويّ والمايكروني باستخدام نبات الصّبار كمعمل حيويّ: وفعاليّتها المضادة لفطور المبيضات البيض والرشاشية السّوداء

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ملخص

صُنعت جسيمات أوكسيد المغنيزيوم النّانويّة والمايكرونية بواسطة صبار التين الشوكي وتم تقييم فعاليتها المضادة للفطور المسببة للأمراض الهامة عند البشر كمبيضات البيض والرشاشيات السّوداء. وتم توصيف الجسيمات الناتجة بِـ UV–Vis، FTIR، DLS، EDX، FESEM. أظهر تحليل مطيافية الأشعة المرئية وفوق البنفسجية قمة عند 300 نانومتر، وبيّن اختبار الأشعة تحت الحمراء أن المستقلبات الحيوية في النبات تلعب دورًا مهمًا في إرجاع أيونات المعدن ثم نموها لتعطي في النهاية جسيماتٍ نانويّة، كما يبين التحليل قمة قريبة من 400 $^{-1}$سم تعود للرابطة Mg-O-Mg. يؤكد تحليل الـ EDX وجود جسيمات أوكسيد المغنيزيوم. تراوحت أبعاد جسيمات أوكسيد المغنيزيوم النّانويّة ذات الشكل الكروي بين 15.5 و 78.01 نانومتر (بمتوسط 42.28 نانومتر)، أما أبعاد الجسيمات المايكرونية الكروية تراوحت بين 105.2 و 1313.9 نانومتر (بمتوسط 356.09 نانومتر) وذلك باستخدام المجهر الإلكتروني الماسح عالي النفاذية FESEM. بينما كان متوسط الأبعاد المحسوب بطريقة التشتت الضوء الديناميكي DLS مساوياً لِـ 46.04 و 377 نانومتر. اختبار الفعالية المضادة للفطور في المختبر *In vitro* للجسيمات الناتجة تمت دراسته بواسطة طريقتين مايكروبيولوجيتين هما الانتشار في الآبار وتمديد المرق الدقيق في كلتا الطريقتين كانت للجسيمات النّانوية تأثير أكبر من الجسيمات بالحجم الأكبرضد الفطور، وكان التركيز المثبط الأدنى الـ MIC لجسيمات أوكسيد المغنيزيوم النّانويّة مساوياً لِـ 1.5 ، 6.25 ملغ / مل لكل من المبيضات البيض والراشاشيات السوداء على التوالي.

الكلمات الدالة: أوكسيد المغنيزيوم النّانويّ، صبار التين الشوكي، التأثير المضاد للفطور، فطور المبيضات البيض، الرشاشية السّوداء.

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Fabrication of Interpenetrating Polymer Network-Based Hydrogel for Colon-Targeted Release of Nateglinide

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ABSTRACT

Nateglinide is an anti-diabetic agent that experiences modest first-pass metabolism and poor aqueous solubility. This paper explores the preparation, characterization, and evaluation of interpenetrating polymer network composite hydrogels of chitosan and poly(meth(methacrylic)) acid as a potential carrier for the drug. Interpenetrating polymer network composite hydrogels of chitosan and poly(meth(methacrylic) acid incorporating nateglinide were prepared using N,N'-methylene bisacrylamide and glutaraldehyde as cross-linkers. The polymerization of chitosan, entrapment of the drug, and its interaction in prepared hydrogels were checked by FTIR spectroscopy, DSC, and powder XRD studies. The hydrogels were evaluated for their swelling behavior and in vitro drug release. The morphology of the hydrogels before and after dissolution was studied using SEM. The hydrogels showed a 93.29 ± 4.65% yield and 91.28 ± 2.22% drug loading. The hydrogels exhibited pH-sensitive swelling behavior. The in vitro release profile confirmed that the drug release depended on the swelling of hydrogels and showed a biphasic release pattern. Chitosan-poly(meth(methacrylic)) acid interpenetrating polymer network hydrogel, with its biodegradable nature and pH-sensitive release of nateglinide, is an attractive option to be further explored for targeted controlled drug delivery formulations for the drug.

Keywords: Chitosan-Poly(meth(methacrylic)) Acid hydrogel; Interpenetrating Polymer Network; Nateglinide; Biodegradable; pH-Sensitive.

INTRODUCTION

Nateglinide (NAT) is used for the treatment of type 2 diabetes. This blood-glucose-lowering drug belongs to the meglitinide class[1]. NAT has been classified to belong to Class II per the Biopharmaceutical Classification System[2] as it has good intestinal permeability but inadequate water solubility of 0.061 mg/mL[3]. In addition to selectively blocking beta-cells of the pancreas, it has a short half-life of 1.5 to 2.5 hours[4]. Rapid intestinal absorption is observed with NAT. In the physiological intestinal environment, where pH is approximately 6.5, the compound is predominantly ionized due to its pKa value of 3.1[5]. Over a dose range of 62 - 240 mg, the drug displays linear pharmacokinetics, with no dose-dependent time to peak concentration[6]. CYP2C9 and CYP3A4 are the primary cytochrome P450 enzymes involved in its biotransformation in the liver [7]. After oral administration of 120 mg of the drug 10 minutes before a meal, peak plasma concentrations of 10.08 g/mL are reached in approximately 0.5 - 1.0 hours[8]. The short half-life and variable bioavailability of this drug may be due to its poor solubility in water, both of which could be improved by sustaining its release over time.

Interpenetrating polymer networks (IPN) have been around since 1914, when Aylsworth invented the first one[9]. IPN refers to a network composed of at least two polymers,

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one synthesized in the presence of the other. A physical crosslinking network is formed when polymer chains in the second system entwine with polymer chains in the first system [10]. Since each polymer retains its unique properties, synergistic improvements in characteristics such as strength or toughness are observed. Additionally, it's important to note that IPNs differ from polymer blends in that they exhibit swelling behavior without dissolution in solvents, and they effectively suppress creep and flow.

As a consequence of exhibiting superior performance compared to conventional individual polymers, Interpenetrating Polymer Networks (IPN) have witnessed a surge in applications. Their advanced properties have garnered significant attention in the pharmaceutical industry, especially in the realm of drug delivery. With the ability to create nontoxic, biocompatible, and biodegradable polymer networks, IPNs are gaining prominence for delivering bioactive molecules, particularly in the context of controlled and targeted drug delivery. The versatility of IPNs is evident through the multitude of molecules produced via this approach, all aimed at enhancing targeting and bioavailability [11–14]. Over the years, formulators have focused fabricated these IPNs into various dosage forms [15–23]. They have also made them smarter by making them respond to stimuli such as magnets [24], temperature [25], pH [26], ions [27], electrons [28], and light [29].

A hydrogel is a polymeric material capable of holding large amounts of water in its three-dimensional network due to its hydrophilic nature[30]. Despite its ability to swell and retain moisture, a hydrogel does not dissolve in water. The hydrophilic functional groups attached to the polymer backbone contribute to its water-absorbing properties, while crosslinking between network chains imparts resistance to dissolution[31]. Hydrogels derived from natural polymers, such as chitosan, have garnered significant attention due to their biocompatibility and the ease of preparing newer derivatives. However, unmodified chitosan faces challenges such as low mechanical strength and limited control over the release of entrapped molecules, stemming from its hydrophilic nature and excellent solubility in an acidic medium. Therefore, using modified chitosan hydrogels for the controlled delivery of bioactive molecules is beneficial [32,33]. One such modification is the formation of Interpenetrating Polymer Networks. Chitosan-Poly(meth(methacrylic)) Acid (C-PMMA) hydrogels are an example of IPN exhibiting good mechanical strength and pH-sensitive swelling behavior [34]. These hydrogels have been explored for the controlled release of drugs like amoxicillin [35], meloxicam [35], and metronidazole [36], to name a few. The current paper investigates the synthesis, characterization, and evaluation of this IPN as a potential carrier for the slow release of NAT.

RESULTS

Characterization of chitosan

Chitosan's molecular weight and N-deacetylation percentage were measured as 3.5×10^5 Da and 84.6%, respectively.

High performance liquid chromatography analysis

It was observed that NAT's retention time was 5.3 min, with a single, sharp peak being obtained. The method was linear in the range of 2.5 to 20 µg/mL. According to the calibration curve, the slope was 80440, and the intercept was -43720. The correlation coefficient was 0.9984.

Synthesis of chitosan-poly(meth(methacrylic)) acid IPN hydrogel, yield calculation and drug loading

C-PMMA IPN was synthesized through a solution co-polymerization/crosslinking reaction, employing potassium persulfate (KPS) as a redox initiator to initiate polymerization. The results, including yield and drug loading for various samples, have been tabulated in Table 1.

Table 1: Yields and drug loading obtained for different formulations of hydrogels

Formulation	Yield (%) n = 3 ± SD	Drug loading (%) n = 3 ± SD
K1	93.29 ± 4.65	91.28 ± 2.22
K2	95.38 ± 2.63	89.95 ± 2.91
K3	94.13 ± 3.46	92.88 ± 3.02
K4	92.58 ± 2.11	93.47 ± 2.78

Swelling studies

IPNs' swelling properties depend heavily on their gel structure, crosslinking density, and surrounding medium composition[37]. The percentage swelling index for the hydrogels under different pH conditions is depicted in **Figure 1**.

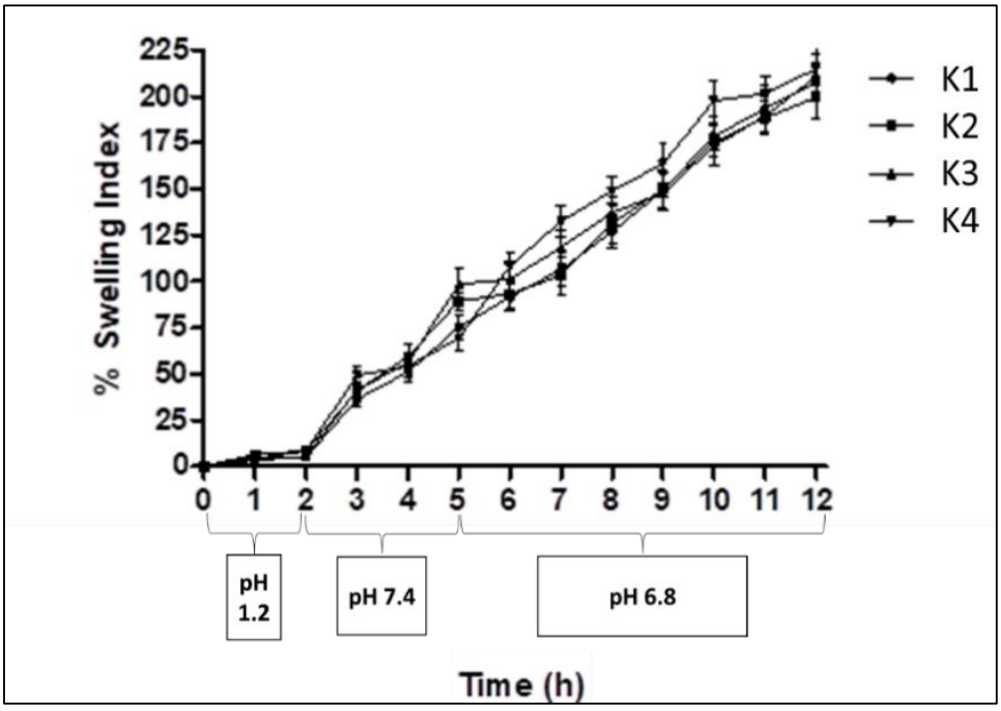

Figure 1. % Swelling Index for C-PMMA IPN hydrogels containing NAT (n = 3)

In vitro drug release studies and release kinetics

Figure 2 shows the cumulative release of NAT from C-PMMA IPN hydrogels at different pH conditions. In all four hydrogels, only K1 released NAT almost entirely after 12 h; therefore, it was considered an optimized formulation.

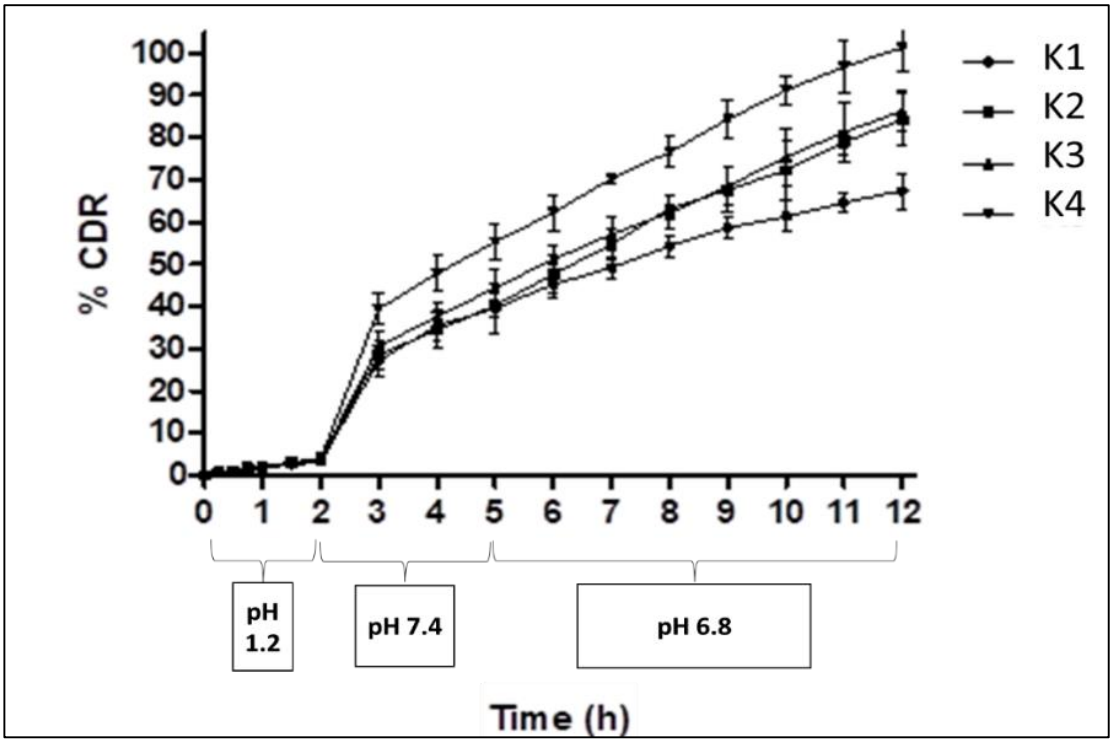

Figure 2. % Cumulative Drug Release (CDR) under different pH conditions for NAT from C-PMMA IPN hydrogel

The release of the drug followed zero-order kinetics (R^2 = 0.9965) for the middle three hours (from hours two to five) when the dissolution media used was phosphate buffer pH 7.4. It displayed Korsmeyer Peppas non-Fickian diffusion (diffusion coupled with erosion) kinetics with n > 0.5 at the higher pH of 6.8 (R^2 = 0.9932, n = 0.6406). According to our studies, the pH-dependent C-PMMA IPN hydrogel system does assist in the controlled release of NAT in the colon, which could contribute to improved oral bioavailability of the drug.

FTIR spectroscopic study

The FTIR spectra of chitosan, C-PMMA IPN hydrogels and NAT are given in **Figure 3**.

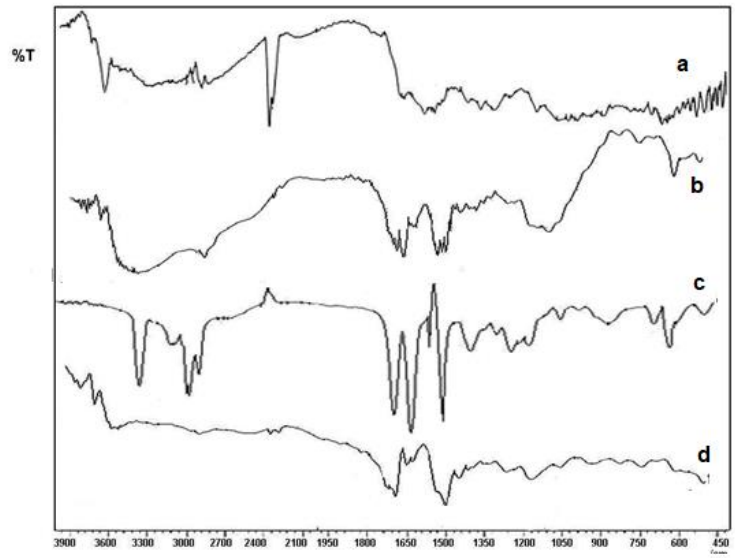

Figure 3: FTIR spectra of (a) Chitosan, (b) blank C-PMMA IPN hydrogels, (c) NAT, and (d) NAT-loaded C-PMMA IPN hydrogels

Differential Scanning Calorimetry study

DSC analysis was performed to characterize the thermal behavior of chitosan, C-PMMA IPN, and NAT in the hydrogel formulation. The endotherms obtained are shown in Figure 4.

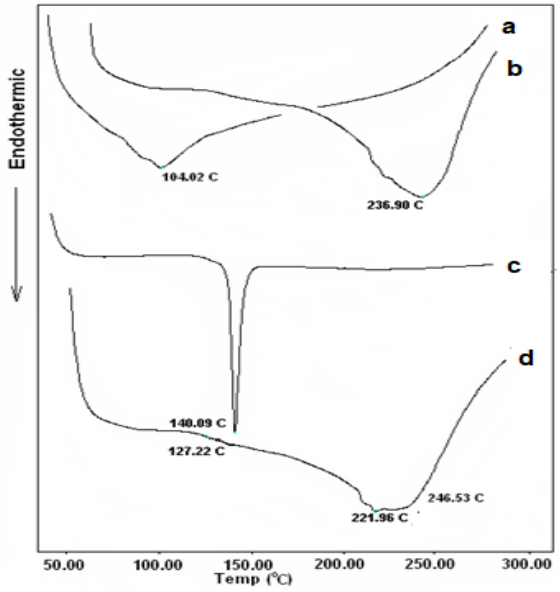

Figure 4: DSC thermogram of (a) Chitosan, (b) Blank C-PMMA IPN, (c) NAT, and (d) NAT-loaded C-PMMA IPN formulation

Powder X-Ray diffractometry study

The X-ray diffractograms of chitosan, blank C-PMMA IPN, NAT, and NAT-loaded C-PMMA IPN are shown in Figure 5.

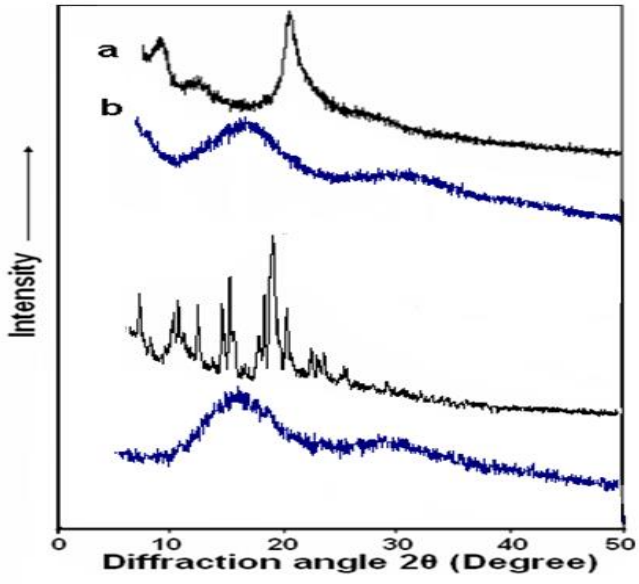

Figure 5: X-ray diffractogram of (a) Chitosan, (b) Blank C-PMMA IPN, (c) NAT, and (d) NAT-loaded C-PMMA IPN formulation

Scanning electron microscopy study

A surface morphology study was conducted on an optimized hydrogel containing NAT before and after dissolution. The surface morphology showed a translucent non-porous membrane (Fig. 6a). Following dissolution, the surface morphology revealed the presence of open channel-like structures (Fig. 6b).

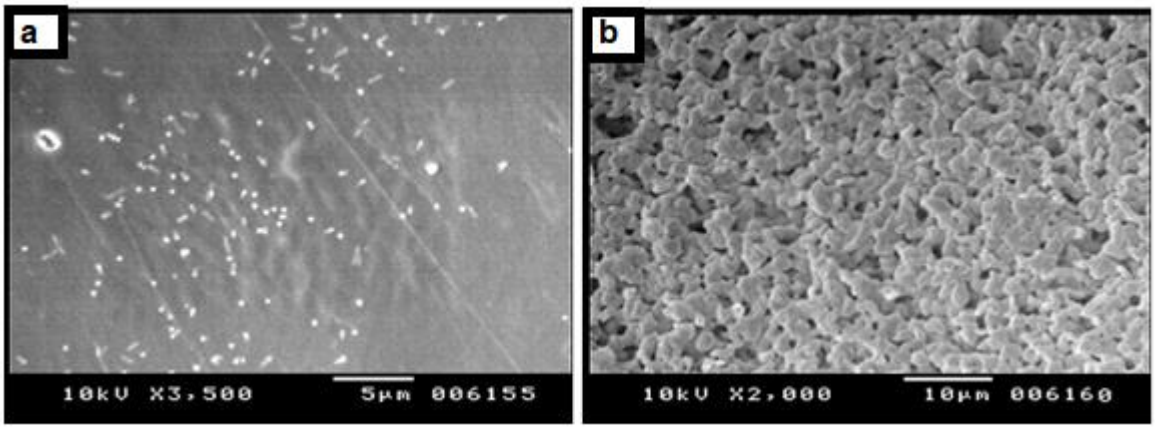

Figure 6: SEM of C-PMMA IPN containing NAT (a) Before dissolution and (b) After the dissolution

Determination of unreacted glutaraldehyde and methacrylic acid

The gas chromatography study conducted to quantify glutaraldehyde levels in the formulations revealed that only 4.1 ppm of the crosslinker remains. Furthermore, the unreacted monomer accounted for 1.54 mg. These two values confirmed that the proportion of unreacted material was small enough not to affect the toxicity or stability of the formulation.

DISCUSSION

Synthesis of chitosan-poly(meth(methacrylic)) acid IPN hydrogel, yield calculation and drug loading

Aqueous solutions of KPS decompose into sulphate ion Aqueous solutions of KPS decompose into sulfate ion radicals, which further react with water to produce hydroxyl radicals. In this scenario, chitosan acts as a weak reducing agent, forming chitosan radicals in response to these hydroxyl radicals, leading to further polymerization propagation[38]. NH_3^+ groups in chitosan and COO^- groups in poly(meth(methacrylic)) acid can interact electrostatically to form polyelectrolytes[39]. As a result of the presence of a solvent, heat is dissipated. After preparing the hydrogels, they were washed with distilled water to remove the monomer, oligomer, crosslinking agent, initiator, soluble and extractable polymer, and other impurities.

Swelling studies

The intrinsic pKa of chitosan is 6.5, below which NH_2 groups are ionized, i.e., they are in the $–NH_3^+$ form. At pH levels above 4.5, carboxylic groups in methacrylic acid become ionized [40]. Due to the combination of anionic and cationic groups, the prepared hydrogels were amphiphilic. Additionally, anions crosslink with chitosan. In acidic conditions (pH = 1.2), acid groups are not ionized, and swelling is mainly controlled by protonated amino groups ($–NH_3^+$) attached to the C2 carbon of the chitosan [41]. Within these hydrogels, poly(meth(methacrylic)) acid might help protonate amines from chitosan, causing electrostatic repulsion between chains of polymers [42]. Due to the polymer's charges, chitosan chains are electrostatically repelled from each other, and a higher osmotic pressure is created inside the gel. Gel swelling balances the difference in osmotic pressure between the network's internal and external solutions [41]. However, the swelling was not observed in the present case due to high crosslink densities due to glutaraldehyde. Poly(meth(methacrylic)) acid ionizes above pH 4.5, resulting in higher swelling. Chitosan amino groups were ionized at pH 6.8, and ionic bonds dissociated. This led to the increased swelling ratio of the hydrogel due to the electrostatic repulsion of COO^- ions from poly(meth(methacrylic)) acid. A similar phenomenon was seen in the ileocecal phase of the study [41]. A hydrogel's maximum swelling level is determined by the balance between contractile and repulsive forces within the network. There is a possibility of the complex dissolving if there is a high level of swelling. If we maximize the number of electrostatic interactions between two oppositely charged polymers, the grade of network complexation will be maximized. As a result, the network will become more stable, reducing swelling/eroding behavior in hydrogels. Due to its tighter structure, the network will be able to control the release of drugs [43]. The C-PMMA IPN hydrogels were made by crosslinking chitosan with glutaraldehyde; therefore, the amino groups present were meagre. Furthermore, the degree of swelling is inversely proportional to the crosslinking; therefore, the degree of swelling would decrease at all pH values compared to plain chitosan hydrogels.

In vitro drug release studies and release kinetics

C-PMMA IPN are hydrophilic that can absorb water and swell in an aqueous environment. When the IPN come into contact with a surrounding medium, such as a physiological solution, they absorb water and increase in size. The swelling of the IPN creates pores and channels within the polymer matrix [12]. As the C-PMMA IPN swell, the drug molecules start to diffuse out of the IPN in response to a concentration gradient. The rate of diffusion

is influenced by factors such as the molecular weight and size of the drug molecules, the degree of cross-linking of the polymer, and the nature of the surrounding medium [44]. NAT is released biphasically in response to pH changes in the hydrogel. The polymer used is a pH-responsive one which is soluble at pH 6 and above. Thus, the polymer did not swell or solubilize at pH 1.2 which mimicked the acidic conditions of the stomach [45]. C-PMMA IPN hydrogels are amphiphilic since they contain both anionic and cationic groups. Chitosan's protonated amino groups were mainly responsible for controlling the swelling of these hydrogels; under acidic conditions, this effect was negligible. As a result, drug release in the first two hours in SGF was also insignificant. However, as pH increased, swelling increased, resulting in an increase in drug release from the hydrogel [46]. In addition to diffusion, the drug release from C-PMMA IPN can also be affected by polymer degradation. PMMA is known to undergo hydrolysis under certain conditions, resulting in the breakdown of polymer chains. The degradation of the polymer can lead to the release of encapsulated drug molecules. The rate of polymer degradation depends on factors such as pH, temperature, and the presence of enzymes or other catalysts [47]. Overall, the drug release from C-PMMA IPN involves a combination of swelling, diffusion, and polymer degradation. These factors collectively contribute to the pH-dependent controlled and sustained release of drugs from the polymer matrix, allowing for precise modulation of drug delivery kinetics [48].

FTIR spectroscopic study

FTIR spectrum of chitosan revealed a broad absorption band in the 3500 and 3100 cm^{-1} range, centered at 3400 cm^{-1}, due to O–H stretching vibration, N-H extension vibration and the intermolecular H-bonds of the polysaccharide moieties (Fig. 3a). Axial stretching of C–H–bonds were observed corresponding to a band at 2881 cm^{-1}. A peak at 1676 cm^{-1} is attributed to the axial stretching of C=O bonds of the acetamide group which indicated that the sample was not fully acetylated. A band at 1557 cm^{-1} owing to the angular deformation of the N–H bonds of the amino group was observed. A band at 1370 cm^{-1} due to the symmetrical angular deformation of CH_3 and the amide III band at 1320 cm^{-1} were also noted. The band in the range 1156-898 cm^{-1} corresponding to the polysaccharide skeleton, including the vibrations of the glycoside bonds, C–O and C–O–C stretching was observed [49,50].

In C-PMMA IPN hydrogels (Fig. 3b), an additional peak around 1720 cm^{-1} was observed which represented carboxylate ion. Negatively charged carboxylate ion and positively charged NH_3^+ co-existed in C-PMMA IPN hydrogels [51]. New peaks at 1530-1540 cm^{-1} were found in the blank IR spectra of C-PMMA IPN hydrogels probably due to the ionic interaction between chitosan and the acids. Glutaraldehyde was used as cross-linking agent for chitosan, hence an additional peak at 1680 cm^{-1} was observed indicating the formation of Schiff's base as a result of the reaction between the carbonyl group of glutaraldehyde and amine group of chitosan chains ensuring the proper formation of the IPNs [52,53].

The FTIR spectrum of NAT (Fig. 3c) revealed peaks at 3184.25 cm^{-1} due to NH stretching; at 3064.68 cm^{-1} and 2948.96 cm^{-1} attributed to alkyl and phenyl stretching, respectively; 2655.68 cm^{-1} due to N^+ stretching; 1685.68 cm^{-1} due to C=O stretching; 1525.59 cm^{-1} and 1385.23 cm^{-1} due to symmetric and asymmetric stretching of NO_2 group; 1470.5 cm^{-1} and 1385.23 cm^{-1} due to bending of geminal methyl group and at 781.12 cm^{-1} and 703.97 cm^{-1} due to out of plane bending of 5 and 3 adjacent hydrogen of the aromatic ring [54]. All these peaks were exhibited in the FTIR spectrum of optimized C-PMMA IPN hydrogel containing NAT also (Fig. 3d), which confirmed the presence of the drug in the hydrogel without any significant alteration of the functional group owing to reaction with other ingredients of the hydrogel.

Differential Scanning Calorimetry study

At 104.02 °C, pure chitosan displayed a broad melting endotherm (Fig. 4a). IPN C-PMMA (Fig. 4b) exhibited melting endotherms at 236.99 °C. Amidation between

chitosan and poly(meth(methacrylic)) acid may explain the higher glass transition temperature (Tg). The carboxylate groups and carboxyl groups in the C-PMMA IPN react when heated to 200 °C, producing amide bonds between the ammonium groups and non-protonated amino groups of chitosan [34]. NAT exhibited a sharp endotherm at 140.90 °C (Fig. 4c), which matches its melting point [55]. In C-PMMA IPN, NAT did not show sharp melting endotherms (Fig 4d), indicating either an amorphous form of the drug or a solid solution.

Powder X-Ray diffractometry study

Chitosan's XRD pattern presented the most intense diffraction intensity in the broad peak between 17 and 23 degrees, which reflected the semicrystalline nature of the material (Fig. 5a), as reported by the literature [56]. According to Figure 5b, there is no sharp crystalline peak in the diffraction pattern of the blank C-PMMA IPN, which indicates its amorphous nature. NAT's diffraction pattern (Fig. 5c) showed characteristic peaks at 8.16°, 11.54°, 13.14°, 15.22°, 15.98°, 19.76° and 21.06°, indicating its crystalline nature [57]. NAT-loaded C-PMMA IPN formulations are devoid of all sharp peaks, as seen in the diffractogram (Fig. 5d). As a result, it was determined that the drug was amorphous in the final formulation.

Scanning electron microscopy study

The open channel-like structures could be due to the ionization of carboxyl groups and dissociation of ionic crosslinks between chitosan and poly(meth(methacrylic)) acid, which is consistent with swelling results.

EXPERIMENTAL SECTION

General Experimental

Methacrylic acid was sourced from Sigma Aldrich, USA. Chitosan was donated by Central Marine Fisheries Research Institute (Cochin, India). NAT was obtained as a gift sample from Zydus Research Centre, Ahmedabad (India). N,N-methylene-bis-acrylamide and Potassium persulfate were purchased from S.D. Fine Chem. Ltd., India and Ranbaxy Fine Chemicals Ltd., India, respectively. All other chemicals and reagents such as acetonitrile, methanol, phosphoric acid used were of analytical grades and used as received.

Characterization of chitosan

In a solvent containing 0.1 M acetic acid and 0.2 M NaCl maintained at 25 °C, the average molecular weight of chitosan was determined by Mark-Houwink viscometry [49].

The degree of N-deacetylation was determined by FTIR using the following relationship:

$$\%N - deacetylation = 100\left[1 - \left(\frac{A_{1655}}{A_{3398}}\right)\left(\frac{1}{1.33}\right)\right] \quad (1)$$

In this case, A corresponds to the absorbance at the given wave number, whereas 1676 and 3400 cm^{-1} correspond to amide and chitosan primary amino groups, respectively. In the case of fully N-acetylated chitosan, factor 1.33 represents the A_{1676}/A_{3400} [58].

High performance liquid chromatography analysis

In this study, High Performance Liquid Chromatography (HPLC) measurements were conducted using Shimadzu's LC 2010 AHT system, equipped with a UV/ Visible detector. We analyzed the samples on a Kromasil C18 column (250 x 4.6 mm ID, 5 m pore size) equipped with an auto integrator. In the mobile phase, 0.067 M monobasic potassium phosphate, acetonitrile and methanol in a 60:30:10 % v/v ratio. The flow rate was 1.0 mL per minute at 50 °C for 15 minutes at a pressure of 1500 PSI. A wavelength of 210 nm was used to detect NAT with a retention time of 5.3 minutes [59]. This study used a calibration curve based on concentrations ranging from 2.5 µg/mL to 20 µg/mL to evaluate all samples (with 20 µL injection volume).

Synthesis of chitosan-poly(meth(methacrylic)) acid IPN hydrogel, yield calculation and drug loading

C-PMMA IPN hydrogels incorporating NAT were prepared using N, N'-methylene bisacrylamide (MBA) and glutaraldehyde as crosslinkers in the presence of redox initiator, KPS. To prepare a methacrylic acid monomer solution, 2.8 g of methacrylic acid were dissolved in 0.1 M

acetic acid (5 mL). Next, we carried out the polymerization reaction in flasks by dissolving 1 g of chitosan in 2% acetic acid (40 mL), followed by adding a monomer solution of methacrylic acid. NAT was added to this solution. To initiate the reaction, KPS (108 mg) was added, followed by MBA (4 mg) and glutaraldehyde (0.15 mL). The polymerization flask was placed in a thermostatic bath at an elevated temperature of 120 °C for one hour. After allowing the mass to cool to room temperature, it was filtered and washed several times with distilled water to remove unreacted chemical contaminants [60]. The formulae for the trials for the preparations of IPN are given in **Table 2**.

Table 2: Formulae for C-PMMA IPN hydrogels

Ingredients	**Formulations**			
	K1	**K2**	**K3**	**K4**
Chitosan (g)	1.0	1.0	1.0	1.0
Methacrylic acid (g)	2.8	2.8	2.8	2.8
KPS (mg)	108	108	108	108
MBA (mg)	4.0	4.0	4.0	4.0
Glutaraldehyde 25% (mL)	0.15	0.15	0.15	0.15
NAT (g)	0.5	1.0	1.5	2.0

The yields of the resultant hydrogels were calculated using the formula:

$$\% \text{ Yield} = \left(\frac{A}{B}\right) \times 100 \quad (2)$$

Where A is the actual weight of the hydrogel and B is the theoretical weight of the hydrogel.

Crushed hydrogel samples containing 10 mg of NAT were used to determine drug loading. After weighing and transferring the hydrogel samples to a 10 mL volumetric flask, the volume was filled with methanol. Keeping the solution aside allowed the insoluble matter to settle. Next, a 10 mL volumetric flask was filled with 5 ml of supernatant clear liquid, which was then diluted with mobile phase to volume and mixed. Using the predefined HPLC method, ten µl of the solution was assayed. The concentration of NAT was calculated, and drug loading was determined using the following formula:

$$\% \text{ Drug Loading } = \left(\frac{X}{Y}\right) \times 100 \quad (3)$$

X is the actual concentration of NAT in the hydrogel, and Y is the theoretical concentration of NAT in the hydrogel.

Swelling studies

Hydrogels were soaked in buffer solutions, and incubated at 37°C under 150 rpm, and their swelling behavior was measured. In the first step, dry hydrogels were immersed in 0.1 M HCl solution at pH 1.2 for two hours (gastric phase). After that, the hydrogels were transferred to a sodium phosphate buffer solution at pH 7.4 and were allowed to stand for three hours (small intestine phase). In the final step, they were transferred to a sodium phosphate buffer solution at a pH of 6.8 for seven hours (colonic phase) [61,62]. Samples were removed from the swelling medium at certain intervals and blotted with filter paper to remove excess moisture. To calculate the percentage of swelling, SI, at each time, we used the following expression [41] :

$$\%SI = \left[\frac{W_t - W_0}{W_0}\right] \times 100 \quad (4)$$

W_t and W_0 are the sample weights at time t and in the dry state, respectively.

In vitro drug release studies and release kinetics

In vitro dissolution studies of NAT from hydrogels were conducted using a USP XXIV dissolution rate test apparatus (type II, model TDT-08 L, Electrolab, Mumbai, India) fitted with a paddle (100 rev/min) at 37 °C. Dissolution was conducted in 250 mL simulated gastric fluid (SGF, pH 1.2) for two hours, in 900 mL phosphate buffer solution (PBS, pH 7.4) for three hours, and then in

900 mL PBS (pH 6.8) for 12 hours. A sample equivalent to 90 mg of NAT was taken for in vitro drug release studies [63,64]. An aliquot of five mL was withdrawn at predetermined times, filtered through a 0.45-µm membrane filter, diluted, and analyzed using the previously described HPLC method. Using the calibration curve, the % CDR was calculated.

Based on the results of in vitro release studies, various kinetic equations were fitted to determine the likely mechanism of the release of drugs from prepared hydrogels. The kinetic models used in this study were zero order, first order, Higuchi, and Korsmeyer-Peppas models. For each model, rate constants were calculated.

Fourier-transformed infrared (FTIR) spectroscopic study

By analyzing the FTIR spectra recorded for chitosan, NAT, C-PMMA IPN, and optimized formulation, we evaluated the changes in polymer structure after forming hydrogels, crosslinking and the drug-polymer interactions. An FTIR spectrophotometer (FTIR-8400 S, Shimadzu, Japan) was used to record the spectra using KBr pellets in a scanning range of 400-4000 cm^{-1} at a resolution of 2 cm^{-1}.

Differential scanning calorimetry (DSC) study

NAT, C-PMMA IPN, and optimized formulation, were scanned using an automatic thermal analyzer (DSC 60, Shimadzu, Japan) equipped with time domain spectroscopy tread line software. The experiments were conducted using sealed aluminium-lead pans. At a scanning rate of 10 °C/min, the samples were heated from 50-300 °C.

Powder X-Ray diffractometry (PXRD) study

The powder X-ray diffraction study was carried out to characterize chitosan, NAT, C-PMMA IPN and the optimized formulation. X-rays were generated using a Philips X'Pert 3040/60 in Almelo, Netherlands, capable of emitting CuKα radiation (λ=1.54178Å). Data was collected in continuous scan mode at 0.01° steps at 2θ in the scanning range of 5–50°.

Scanning electron microscopy (SEM) study

To examine the surface morphology of the optimized formulation, scanning electron microscopy was used before and after dissolution. A dried film was first mounted on a stub using double-sided adhesive tape. Then, a scanning electron microscope (JEOL, JSM-5600 LV, Japan) was used to observe the films after they had been coated with gold to determine their surface characteristics.

Determination of unreacted glutaraldehyde and methacrylic acid

Gas chromatography was used to determine the amount of unreacted glutaraldehyde. To prepare the standard solution, glutaraldehyde (100 mg) was diluted to 10 mL with N, N-di-methyl pyrrolidone (NMP). First, a 0.2 mL sample was diluted to 10 mL with NMP, after which 0.1 mL was withdrawn and further diluted to 10 mL with NMP. A volume of 5 mL of the resulting standard solution was injected into a gas chromatograph (2014, Shimadzu). One gram of blank C-PMMA IPN was dissolved in five mL NMP and injected into the headspace of a gas chromatograph. The amount of unreacted glutaraldehyde was obtained using the following equation:

$$\frac{AUC_{sample}}{AUC_{std}} \times \frac{0.1}{10} \times \frac{0.2}{10} \times \frac{0.1}{10} \times \frac{5}{1} \times 10^6 \quad (7)$$

The titrimetric analysis determined the amount of unreacted methacrylic acid[65]. A blank formulation containing C-PMMA was immersed in 100 mL of distilled water for 48 hours to dissolve the unreacted monomer. Afterward, it was filtered and diluted with distilled water to a total volume of 100 mL. One drop of phenolphthalein was added to this solution, and it was titrated against a 0.001 N sodium hydroxide solution. Each milliliter of NaOH was equated with 0.08609 mg of methacrylic acid.

CONCLUSION

NAT was entrapped in the IPN hydrogel by crosslinking chitosan with poly(meth(methacrylic)) acid without significant changes in its chemical composition, as evident from the characterization of the hydrogel. In vitro drug release studies and swelling studies of hydrogels

show a direct correlation between swelling and drug release from the hydrogel. The NAT hydrogel delivers its drug biphasically from the hydrogel, with minimal drug release under acidic conditions in the stomach, followed by continuous release under alkaline conditions in the colon. Consequently, this hydrogel would be ideal for releasing NAT into the colon after bypassing the stomach. As NAT is delivered to the colon specifically, the drug's metabolism would be reduced to a great extent, which is the leading cause of the low bioavailability of NAT. Accordingly, we conclude that the C-PMMA IPN hydrogel may be an effective carrier for NAT to increase its oral bioavailability. Because hydrogels are nontoxic and biodegradable, they represent a promising drug delivery system as a cost-effective, straightforward dosage form.

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تصنيع هلام قائم على الشبكة البوليمرية المتداخلة لإطلاق ناتيغلينيد المستهدف للقولون

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ملخص

ناتيغلينيد هو عامل مضاد لمرض السكري الذي يعاني من هضم متواضع في المرور الأول وذوبان مائي ضعيف. تستكشف الورقة الحالية إعدادًا وتوصيفًا وتقييمًا لشبكات البوليمر المتداخلة المركبة للهيدروجيلات المركبة من الكيتوزان وحمض البولي (الميثاكريليك) كحامل محتمل للدواء. تم تحضير شبكات البوليمر المتداخلة المركبة للهيدروجيلات من الكيتوزان وحمض البولي (الميثاكريليك) التي تتضمن ناتيغلينيد باستخدام N,N'- ميثيلين بيساكريلاميد وغلوتارالديهيد كعوامل ربط. تم فحص البلمرة من الشيتوزان ، فخ الدواء وتفاعله في الهلاميات المائية المعدة عن طريق التحليل الطيفي FTIR، DSC ومسحوق XRD الدراسات للمسح الضوئي للمسح الضوئي للمسح الضوئي. تم تقييم الهيدروجيلات لسلوكها في الانتفاخ وإطلاق الدواء في الكشف الخارجي. درست مورفولوجيا الهيدروجيلات قبل وبعد الانحلال باستخدام. SEM أظهرت الهيدروجيلات نسبة عائد تبلغ 93.29 ± 4.65 % ونسبة تحميل الدواء تبلغ 91.28 ± 2.22 %. أظهرت الهيدروجيلات سلوك انتفاخ حساس لقيم ال .pH أكد ملف الإطلاق في المختبر أن إطلاق الدواء يعتمد على انتفاخ الهيدروجيلات وأظهر نمط إطلاق ثنائي الطور. هلام الكيتوزان–حمض البولي (الميثاكريليك) المتداخل للشبكة البوليمرية مع طبيعته القابلة للتحلل والإطلاق الحساس لقيم ال pH لناتيغلينيد خيار جذاب يجب استكشافه بشكل أوسع لصياغات توصيل الدواء المستهدفة والمحكومة للدواء.

الكلمات الدالة: هلام الكيتوزان–حمض البولي (الميثاكريليك) ؛ شبكة البوليمر المتداخلة ؛ ناتيجلينيد ؛ قابلة للتحلل ؛ حساس لقيم ال .pH

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Quality by Design Approaches in Pharmaceutical Development and Solid Dosage Forms Production: A Narrative Overview

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ABSTRACT

Quality by Design (QbD) is an essential approach to pharmaceutical development and manufacturing that has garnered significant attention in recent years. Quality in services, products, and procedures equates to customer satisfaction. Consequently, it facilitates a transition in the pharmaceutical sector and the Food and Drug Administration (FDA) toward a more scientific, risk-based, comprehensive, and proactive drug development strategy. The pharmaceutical industry is actively seeking new solutions to ensure product quality and efficiency. This paper provides a comprehensive overview of QbD principles and their application in the pharmaceutical industry. The benefits of implementing QbD principles are discussed, encompassing increased efficiency, reduced costs, and improved product quality, safety, and efficacy. As the pharmaceutical industry continues to evolve, QbD will remain a crucial aspect of drug development and manufacturing. This article aims to provide pharmaceutical professionals with a comprehensive understanding of the QbD approach.

Keywords: Quality by Design (QbD), Pharmaceutical Development, Solid Dosage forms, Drug manufacturing, Risk-based approach.

1. INTRODUCTION:

Quality by Design (QbD) is defined by the International Conference on Harmonisation of technical requirements (ICH) for registration of pharmaceuticals for human use as "a systematic approach to pharmaceutical development that begins with established objectives and stresses product and process understanding and process control, based on strong science and quality risk management"(1). Along with safety and efficacy, quality is a primary need for a substance to be considered for drug qualification and approval (2). Recent efforts have focused on constructing "quality," as opposed to merely testing it, to guarantee the reliability of pharmaceutical products and systems. Originally proposed by Juran and Godfrey in 1998 (4) , QbD integrates quality into processes using optimisation strategies to understand and control system variables, an approach gaining momentum in the auto industry and recently endorsed by the FDA for pharmaceuticals (3-5).

QbD prioritizes the creation of an optimal process and a thorough understanding of performance for the desired product result. This approach hinges on continuous improvement based on process insights, aiming for a 'desired state' of 'regulatory flexibility'. It emphasizes scientific knowledge development, superior design, performance demonstration, and integrates Quality Risk

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Management (QRM), Design of Experiments (DoE), and Process Analytical Technology (PAT) for continuous learning and lifecycle management (6).

Janet Woodcock, Director of the Centre for Drug Evaluation and Research, defined a high-quality pharmaceutical in 2004 as one that consistently delivers the claimed therapeutic benefit to the user (7), and therefore, is free of contamination. In this context, 'quality' is a metric for assessing 'excellence in manufacturing,' characterized by a lack of flaws, deficiencies, and substantial variances. Notably, QbD for generic pharmaceuticals is discussed. A fundamental principle of QbD, as stated in the ICH Q8 guidance, is that "quality cannot be tested into products; it should be built in by design" (8). This study focuses on the use of QbD in ensuring pharmaceutical quality for solid oral dosage forms of small molecules. In the drug development process, the pharmaceutical industry must demonstrate the safety and efficacy of the new drug in accordance with government regulations. The adoption of a novel drug development strategy can potentially bring about significant financial and operational benefits across the entire product life cycle.

Given the recent industry shift toward QbD-based submissions, this article explores the procedures for developing a market formulation and the necessary supporting data. It outlines the steps that should be taken at the beginning of drug development, pre-manufacturing, and market entry attempts. The paper provides a foundation for manufacturing facility needs and outlines the types of data required to address regulatory matters. It also introduces advanced tools that complement the QbD approach. In this study, we present a streamlined, universal method for identifying essential metrics, material attributes, environmental factors, and quality characteristics (9). The paper also explains the risk-based distinctions controlling the assignment of criticality, which is crucial for ensuring uniformity and facilitating the incorporation of QbD concepts into the design of pharmaceutical production processes. The paper's goal is to provide an approach and technical mechanism for developing and deploying a control strategy—a predetermined group of controls based on current knowledge of the product and the manufacturing process designed to guarantee the reliability of both. Control strategy creation is a methodical procedure that requires collaboration among professionals from various fields to bridge the gap between drug research and manufacturing. The actual application of the early Control Strategy is not the focus of this study, but rather the methodologies and principles that went into its creation. Within the context of the product quality lifecycle, this article details the development of the Design Space (10, 11). Over the established design space for materials, process parameters, environmental, and other factors, precise and reliable predictions of product quality attributes are possible. In this paper, we will examine the more technical aspects of creating a Design Space (12).

1.1. Product and Process understanding and QbD:

Effective management of product variability is essential for maintaining consistent product quality. Three principles can guide this endeavor. First, a product's variance is regulated more by its production process than by the product itself. It is feasible to reduce product variability by managing and regulating raw materials, production parameters, and environmental conditions. Second, regulating inputs that affect product quality, such as raw materials and processing parameters, within a specific range of permissible fluctuation can enable precise and trustworthy predictions of product quality features. Finally, while adhering to quality risk management concepts, product performance must be understood across material attributes, manufacturing process alternatives, and process factors. By identifying and justifying important sources of variability, a quality risk management plan may be designed to successfully manage product variability and ensure consistent product quality (13-15).

1.2. Management of the Product life cycle and Iterative Improvement:

Numerous opportunities arise during a product's lifecycle to enhance its quality, providing manufacturers with a great deal of leeway in how they choose to do so. To maintain the highest standards of quality, it is common practice to track how well a procedure is working. Regular production yields a variety of new data and knowledge that may be utilized to refine processes (16). For consistency's sake, businesses often stick with tried-and-true techniques, such as the frozen method for handling their styles, as shown in Figure 1 (17).

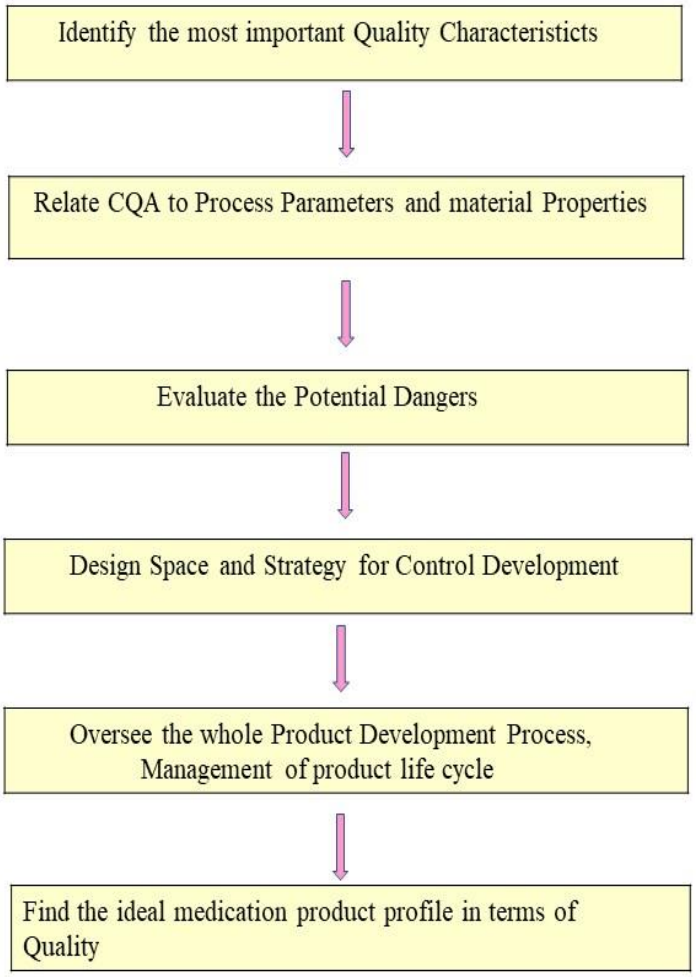

Figure 1: Management of QbD

1.3. Components of an Effective Method:

An effective method, often referred to as a mechanical method, delivers the intended satisfactory results. The selection of clear procedures and test parameters, i.e., components, is crucial for implementing such an effective method (16).

The components of effective method include:

- Controls for the Procedures and
- Controls for the Actual Processing of the Procedures
- Batch-release testing

- Characterisation
- Comparability tests
- Tests for Consistency
- QbD' s approach to risk management emphasises the use of CQAs

1.4. Elements of QbD:

The elements of QbD are as shown in Figure 2 (18).

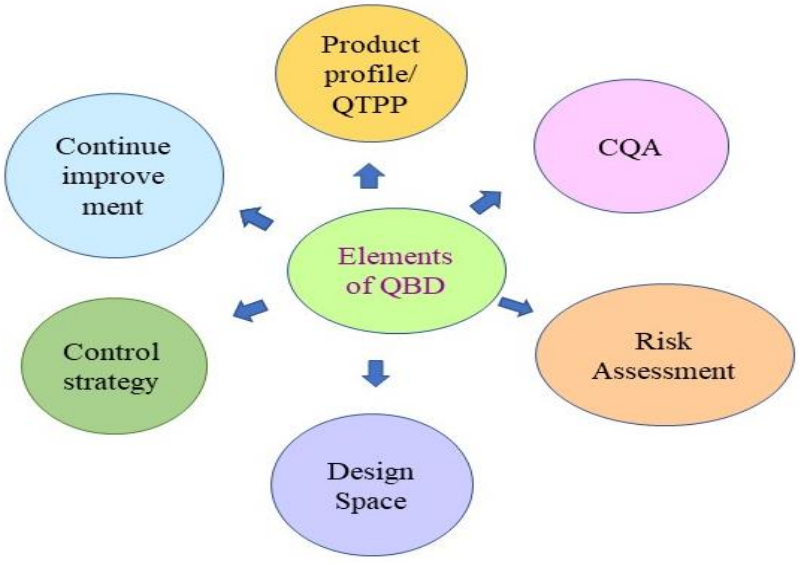

Figure 2: Elements of QbD

These Elements of a Quality-based Design strategy include:

- The TPP can be improved now that links between quality, safety, and efficacy are possible. The norms of the product are established as a baseline for further development and planning.
- Features of Critical Importance Attributes are a type of property that must fall inside certain bounds, ranges, or distributions (19).
- In Risk assessment, critical process parameters and material attributes are compared to critical quality attributes. Risk assessment instruments, such as the FMEA and the bone diagram, will be used to establish the CPPs (20). The planned methods of risk management are detailed in ICH Q9.
- The use of experiment style allows for the establishment and representation of a crucial link between CQAs and CPPs in a stylistic domain (DOEs).
- Long-term planning for the organization: the sooner a problem is isolated and resolved after an unexpected occurrence, the better (21).
- Quality management and maintenance of the product's lifespan (22) and Quality management systems for QbD goods at every step of their lifespan can be established, maintained, and improved using the ICH-Q10 standard.

1.5. Target Product Profile (TPP):

The TPP details the required format of a drug's label and packaging for use in research and development.

1.5.1. Target quality product profile (TQPP):

This is a description of the product's intended application, targeted audience, administrative route, and other essential features and quality design. For a further discussion of product quality, the term TQPP may be a natural progression

from the word TPP. To understand and track information that cannot be orally transmitted from one generation to the next, the QTPP is necessary. To do this, it is necessary to define the qualities an ideal drug should possess while also considering the risks associated with using that drug (23). Quantity, purity, potency, instrumentation closing system, and individuality are all aspects of TQPP that can be produced in any quantities.

1.5.2. Considerable Quality Attributes (CQA):

There are many contexts in which CQA can be used to guarantee the reliability of a product's quality, security, effectiveness, and stability (Certificates of Conformity, or CQA for short). The quality of the result can be defined, measured, and tracked to ensure it stays within acceptable bounds. Quality characteristics consist of clinically safe and effective performance, as well as parameter boundaries nearing failure. Productivity in production is a hallmark of quality (24).The criticality risk could increase if the APT manufacturing process becomes more complex.

1.5.3. Considerable Material Attributes (CMA):

If a genuine change in a parameter makes it impossible for a product to meet a QTPP, then the product must fail. When selecting which characteristics are most significant, it is useful to consider one's level of flexibility and the specificity of each input material. Ranges of CMAs that are considered safe for use in the pharmaceutical industry include active pharmaceutical ingredients, inactive pharmaceutical ingredients, and excipients (24).

1.5.4. Critical Process Parameters (CPP):

It refers to all observable variables involved in the execution of a given procedure step that must be controlled to guarantee the desired result. One could think of each thing in this read as a parameter to a certain method (25). So, here is how it would go down: Prior to or throughout processes that significantly affect the final product's visual appeal, purity, and/or yield, relevant parameters are analyzed for their potential effects as shown in figure 3 (24).

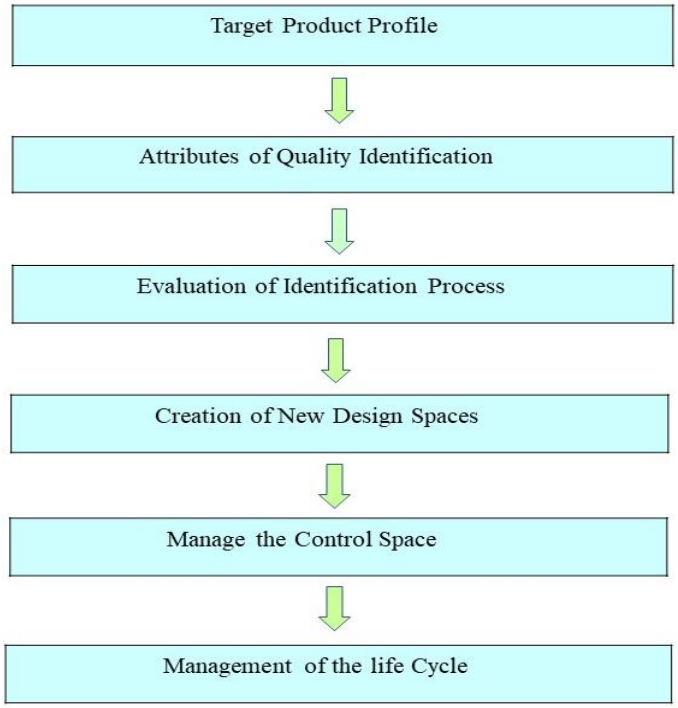

Figure 3: Important Aspects of QbD

1.6. Methods of regulation:

A comprehensive approach to producing high-quality products involves the integration of raw material specifications, process controls, and final product testing. This strategy provides a wealth of data on the substance and process under investigation. PAT serves as a versatile tool within this approach, adapting well to the context in which it is applied (18).

2. DESIGN OF EXPERTS (DOE):

The DOE is a versatile statistical tool leveraged in the design, development, and optimization of various systems, processes, and products. Its wide range of applications includes experimental design, variable screening, transfer function determination, design optimization, and robust design creation (26). In essence, DOE serves as a flexible tool for identifying influential inputs on outcomes in various contexts(27).

2.1. Software for the DOE:

Utilizing efficient statistical software, DOE may be rapidly conceived of and studied. There are paid and open-source statistical programs available for this task (28).There are a variety of popular commercial bundles, like programs that will be employed to carry out DOE are:

- Minitab
- SAS
- SPSS
- Prisma
- Statistica
- Statgraphics

2.2. Benefits of DOE:

Experimental design, or DOE, aims to elucidate the relationship between an independent variable and a dependent variable by gauging the effects of changing the independent variable. This approach requires modulating the independent variable while controlling or minimizing confounding factors. Offering superior control over these factors compared to other methodologies, experimental design enables the replication of results under the same conditions, fostering trust and confidence in the findings. Consistency in outcomes enhances the reliability and validity of the study. With meticulously designed and executed experiments, researchers can robustly examine causal relationships and draw substantive conclusions (29).

2.3. Methods of DOE:

2.3.1. Design Space:

The multidimensional interplay of input variables, including material attributes and process parameters, within the design space guarantees quality. Post-approval modifications are complex and require relocation outside the designated design space. These modifications are subject to regulatory inspection and approval (30). The researcher's individual style may influence the relationship between Y and F, which is a function of the method parameters and quality/material attributes.

2.3.2. Process Analytical Technology (PAT):

It is a state-of-the-art tool for designing, analyzing, and controlling the manufacturing of pharmaceuticals by monitoring critical quality and performance attributes of raw and in-process Materials & processes in real time (i.e., on line, off line, in line) to guarantee the final product meets specifications (31). The idea behind PAT is to minimize potential for harm during the production of a medicine.

2.3.2.1. PAT Implementation at Various Stages:

These are the four stages for the implementation of PAT(32).

- The first step is the collection of data about the manufacturing process.
- Data evaluation of process parameters constitutes the scale-up phase.
- The process understanding phase is temporary.
- Actual Process monitoring and control is the final, permanent Step.

2.3.2.2. Advantages of PAT in Terms of Quality:

To obtain Quality PAT technology is incorporated (18). It had advantages which are listed below:

- For the purpose of incorporating quality into

manufacturing processes

- Effects and variables in the preparation process that influence the final product quality are correlated. There are many factors to consider, such as the surrounding environment, manufacturing methods, and the raw materials.
- Creates a reliable plan for minimizing any damage to the process.
- PAT is used to enhance the manufacturing process all through the product's lifespan.

2.3.2.3. Benefits of PAT:

PAT provides numerous benefits in product manufacturing, including reduced rework, accurate process control, increased safety through automation, and quicker validation processes. These advantages help to reduce errors and improve the overall quality and efficiency of the product manufacturing process (18).

2.3.3. High Quality Risk Management:

The FDA defines risk management as "a strategic safety program designed to lower product risk by using one or more actions or technologies," which is a pretty good definition of what quality risk management is. It is a methodical procedure used to monitor and analyze potential threats to a drug's quality at every stage of development as per the ICH for Q9 guideline (33).

The process of risk management is performed for risk assessment as shown in Figure 4.

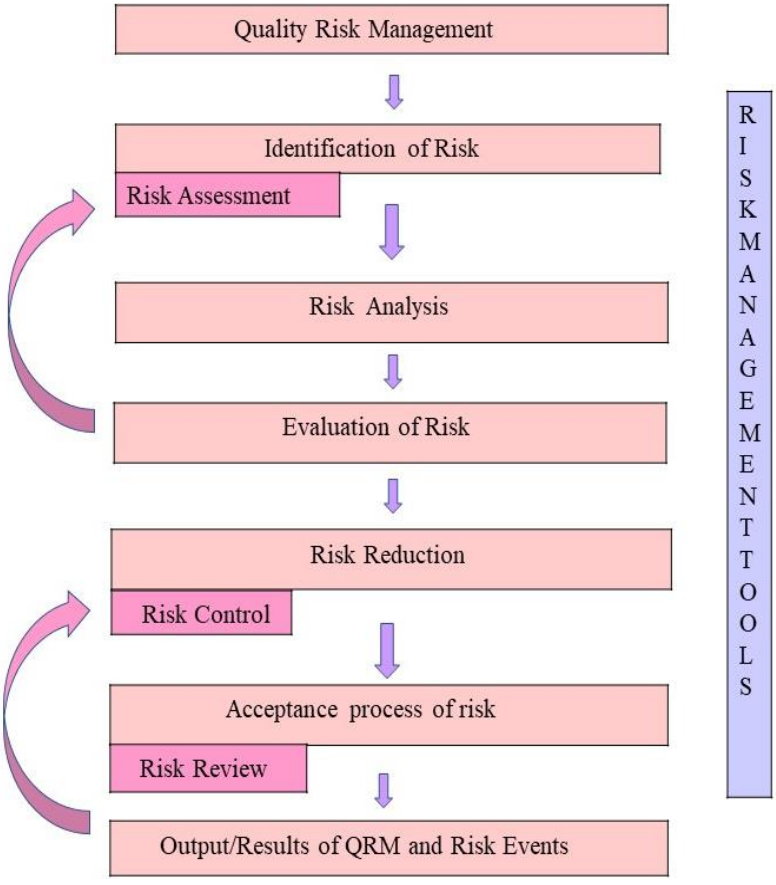

Figure 4: Process of Risk Assessment (17)

2.3.3.1. Probability and Risk analysis:

The term "risk" encompasses not just the potential for harm but also the degree of such harm. The overall quality of a method or procedure can be enhanced through careful consideration of the hazards involved. The purpose of a risk analysis is to determine which factors have the greatest bearing on the final product's quality. When the FDA, commerce, research and development (R&D), prototypes, and numerous manufacturing sites are involved, a risk assessment can facilitate better communication among them all (34).

Here are several ways that risk can be assessed: ICH guideline Q9 outlines a handful of approaches to risk analysis:

- Analysis of Potential Effects of Failures (AFPA)
- Fault Mode, Effects, and Criticality Analysis (FMECA)
- Fault Tree Analysis (FTA)
- Hazard Analysis and Critical Control Points (HACCP)
- Hazard Operational Analysis (HOA)
- Preliminary Hazard Analysis (PHA)
- Risk Ranking and Filtering (RRF).
- Tools in the field of applied mathematics

2.3.3.2. Evaluation of Risk Methods:

➢ **Failure mode effects analysis (FMEA):**

In the pharmaceutical sector, FMEA is a popular method for evaluating potential dangers. It is an approach that anticipates potential problems and systematically works to address them. The term "failure mode" is used to describe any type of problem that can occur in a product, service, or manufacturing system. After identifying potential sources of malfunction, FMEA software ranks the severity of each issue and recommends solutions (19).The criticality of outcomes can be studied using this tool, and it can also provide you a clear picture of the current state of affairs.

➢ **Failure Mode, Effects and Criticality Analysis (FMECA):**

Extending FMEA methodology, we have FMECA. Failure Mode, Effects, and Criticality Analysis is an expansion of FMEA that investigates the severity, frequency, and detectability of potential outcomes. In FMECA, all potential points of failure are cataloged and ranked according to their severity. It is necessary to take corrective action if the danger associated with this criticality is unacceptable. This has applications in areas where there is a high probability of error, such as manufacturing (19). In addition to contributing to control plans and other quality assurance procedures, the tool can be used to design and optimize maintenance plans for repairable systems.

➢ **Fault Tree Analysis (FTA):**

Assuming that a product's or process's functionality would fail, fault tree analysis (FTA) is used. The findings are graphically depicted as a tree of failure modes (19). This can be use in the investigation of a complaint or deviation to determine its cause and verify that any proposed solutions won't introduce new difficulties.

➢ **Hazard Analysis and crucial control points (HACCP):**

It is an organized process that ensures product quality and safety by identifying, managing, and preventing hazards associated with product design, development, and production. HACCP provides extensive documentation to demonstrate process or product awareness by specifying control and monitoring criteria. A hazard is defined as a concern for both safety and quality in a process or product (35).

3. QBD APPROACHES:

Defined objectives, the number of input elements, and interactions to be researched, as well as the statistical validity and effectiveness of each design, should all be considered when choosing the appropriate experimental design. To help clarify the practical applications of Design of Experiments (DoE), experiments can be categorized into two broad groups.

3.1. Screening:

These screening strategies are the most used because of

their low cost. These experimental layouts make it possible to examine a large variety of inputs while keeping the total number of tests to a minimum (36). However, there are some caveats that should be considered if we are to gain a more complete picture of how input factors affect response outcomes.

3.1.1. Two-level full factorial design: A two-level complete factorial design is the most effective screening design since it allows for the estimation of primary effects of input components and their interactions on output responses. The huge number of experiments required is the primary drawback of two-level full factorial designs when compared to fractionate factorial and Placket-Burman designs (37). For a full factorial design with two levels, where k is the total number of inputs, conducting 2K experiments is warranted.

3.1.2. Fractional factorial design:

For screening purposes, fractionate factorial designs are popular because they allow for the assessment of a high number of input elements with a minimal number of experiments.

The formula L^{F}-1 is the foundation of this plan (38). A formulator with three levels and four factors will have thirty-three iterations if the corresponding equation, L^{F}-1= 3^{4}-1=3^{3} =27, is used. Picking the best process run from among these 27.

3.1.3. Placket-Burman design:

Since the Plackett-Burman (PB) design examines the interplay between two variables, it is commonly employed to conduct PB. The PB layout can process 11 variables all at once. This layout was designed for economic efficiency (38).

Two-level fractionate factorial designs (resolution III), such as Plackett-Burman designs, permit investigation of N input components with N experiments (The value of N must be a multiple of 4).

3.2. Optimization:

Because they can simulate a wide variety of response surfaces, these optimization designs are the most used. Screening designs feature only two levels for each input factor, which limits them to simulating a 1st order (linear) response surface. To represent a 2nd order (quadratic) response surface, optimization designs often require 3–5 levels for each input factor.

3.2.1 Three-level full factorial design:

Since a larger number of experiments are needed, a three-level complete factorial design is often employed only when a study of two or three input components is warranted (37).There should be 3k experiments, where k is the total number of inputs being evaluated.

3.2.2. Central Composite Design (CCD):

One of the most common optimization designs is the central composite design (CCD), which requires a lot less tests than the more common three-level complete factorial designs(36).

3.2.3. Box-Behnken design:

The Box-Behnken design, a subset of the three-level fractional factorial design, facilitates the modelling of both first and second-order response surfaces. It provides a cost-efficient alternative when there's an abundance of input variables, as compared to three-level complete factorial designs. Distinctly, the Box-Behnken Design is not a component of factorial or fractional factorial; it operates independently as a quadratic layout (29). Additionally, these designs can be rotated (or near rotated) and exhibit three unique levels for each variable.

4. APPLICATIONS, ADVANTAGES AND DISADVANTAGES OF QBD

4.1. Applications:

4.1.1. Inclusions for FDA Submissions Should Utilize QbD

Adherence to principles is a fundamental aspect of Approval. Here are the noted applications (36):

1. Establishment of a stronger scientific foundation for evaluation.
2. Enhancement of coordination among reviews, compliance checks, and inspections.
3. Improvement in the quality of data submitted to

regulatory agencies.

4. Increased consistency across processes and outcomes.
5. Elevation of evaluation standards by establishing a Quality Management System for Chemistry Manufacturing and Controls.
6. Enhancement of decision-making flexibility.
7. Ensuring that conclusions are based on scientific evidence rather than anecdotal accounts.
8. Inclusion of various fields in the decision-making process.
9. Focused efforts toward the most critical risks.

4.1.2. Utilizing QbD with various analytical approaches (2):

QbD applicable for Analytical Methods (2).

1. Chromatographic methods like HPLC, employed in stability studies, method development, and detection of impurities in pharmaceuticals.
2. Hybrid techniques such as Liquid Chromatography-Mass Spectrometry (LC-MS).
3. Advanced methodologies including ultra-high-performance liquid chromatography (UHPLC) and capillary electrophoresis.
4. Moisture content determination *via* Karl Fischer titration.
5. Compound identification and quantification using UV or other vibrational spectroscopy techniques.
6. Genotoxic residue examination.
7. Dissolution study methodologies

4.2. Advantages of QbD implementation in Pharmaceutical Industry:

Implementing QbD principles holds several benefits across various areas, despite recognized obstacles to its practical deployment (39-42). Understanding these challenges is crucial for regulatory bodies like the MHRA, striving for broader QbD implementation.

These benefits include:

1. Improved quality control (QC) for pharmaceuticals and reduction in product variation through the establishment of a robust approach and minimization of variabilities.
2. Enhanced financial efficiency and reduced regulatory burden due to streamlined manufacturing processes and changes within the "Analytical Design Space" not being considered method shifts.
3. Increased transparency leading to improved comprehension of drug control strategies, thereby expediting the approval, scale-up, validation, and commercialization process.
4. Mitigation of penalties, product recalls, and out-of-specification results through Quality Risk Management (QRM) and continuous improvement mechanisms.
5. Optimization of regulatory scrutiny resulting in more chances of first-cycle approval, reduction in review time for CMC, and updates to regulatory filings.
6. Enhanced probability of successful method transfers from R&D to QC, allowing for the development of novel methods throughout a product's lifecycle.

4.3. Challenges:

There is a need to use QbD to enhance the quality of pharmaceuticals; however, this is often met with resistance due to a lack of familiarity with the industry. There has never been more emphasis on the end result than on a solid scientific understanding of the manufacturing process in the pharmaceutical sector. The pharmaceutical industry strongly endorses adopting QbD. The FDA has asked that the final regulation include terminology criteria for evaluating control systems and standards for switching out analytical approaches. There are ten key barriers that prevent QbD from becoming widespread. The significance of each of these factors depends on the particular medicine at hand and the stage of its adoption process (24, 30).

International organizational barriers include:

- Cross-functional misalignment (e.g., disconnects

between R&D and manufacturing, or quality and regulatory functions).

- Uncertainty regarding QbD implementation costs and timelines.
- Incomplete understanding of CQA implications due to insufficient execution technology (e.g., data management issues).
- The challenge of ensuring suppliers and contract manufacturers align with QbD implementation.

The regulatory authority is actively involved (24, 30) with the next six difficulties:

- Ambiguity in handling QbD applications.
- Unease due to perceived unmet promises of regulatory benefits.
- Inadequate collaboration among international regulatory bodies.
- Insufficient industry support for QbD.
- Limited familiarity with the pharmaceutical process, hindering QbD implementation.
- Difficulty achieving consensus between field inspectors and the FDA on the QbD approach.
- Industry demand for more comprehensive QbD guidance.
- Concern that QbD might protract regulatory approval or yield irrelevant data.

Pharmaceutical businesses believe that QbD may lengthen the time it takes to submit a regulatory approval application or provide the regulatory body with irrelevant information for decision-making.

CONCLUSION:

Quality by Design (QbD) is a vital approach to pharmaceutical development and manufacturing that aims to ensure products consistently meet required quality standards. By implementing QbD principles, pharmaceutical companies can reduce costs, increase efficiency, and enhance product quality, safety, and efficacy. QbD emphasizes a methodical and scientific approach to drug development, including the identification of critical quality attributes and critical process parameters, risk assessment, and continuous monitoring and improvement. Ultimately, QbD can help ensure that patients receive safe and effective medications that meet their healthcare needs. As the pharmaceutical industry continues to evolve, QbD will remain a crucial aspect of drug development and manufacturing.

ABBREVIATIONS:

1. QbD	:	Quality by Design
2. ICH	:	International Council for Harmonization
3. FDA	:	Food and Drug Administration
4. Q8	:	Pharmaceutical Development
5. Q9	:	Quality Risk Management
6. Q10	:	Pharmaceutical Quality System
7. TPP	:	Target Product Profile
8. QTPP	:	Quality Target Product Profile
9. CQA	:	Considerable Quality Attributes
10. CMA	:	Considerable Material Attributes
11. DOE	:	Design of Experts
12. PAT	:	Process Analytical Technology
13. IPQC	:	In - Process Quality Control
14. FTA	:	Fault Tree Analysis

15. FMEA	:	Failure Mode Effect Analysis
16. HACCP	:	Hazard Analysis and Critical Control Points
17. QMS	:	Quality Management System
18. QRM	:	Quality Risk Management
19. RPN	:	Risk Priority Number
20. PBD	:	Placket Burman Design
21. CCD	:	Central Composite Design
22. BBD	:	Box Behnken Design
23. FFD	:	Fractional Factorial Design
24. CMC	:	Chemistry Manufacturing and Controls
25. AFPA	:	Analysis of Potential Effects and Failure
R&D	:	Research and Development

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مناهج التصميم بالجودة في تطوير الأدوية وإنتاج الأشكال الصيدلانية الصلبة: نظرة عامة سردية

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ملخص

التصميم بالجودة ((QbDهو نهج أساسي في تطوير الأدوية وتصنيعها، وقد حظي باهتمام كبير في السنوات الأخيرة. الجودة في الخدمات والمنتجات والإجراءات تعني رضا العملاء. نتيجة لذلك، فهي تسهل الانتقال في القطاع الصيدلاني وإدارة الغذاء والدواء ((FDAنحو استراتيجية تطوير الأدوية التي تكون أكثر علمية، قائمة على المخاطر، شاملة، واستباقية. تسعى الصناعة الصيدلانية إلى حلول جديدة لضمان جودة المنتج وكفاءته. يقدم هذا البحث نظرة عامة شاملة على مبادئ QbD وتطبيقاتها في الصناعة الصيدلانية. يتم مناقشة فوائد تطبيق مبادئ QbD، بما في ذلك زيادة الكفاءة، وتقليل التكاليف، وتحسين جودة المنتج، والسلامة، والفعالية. مع استمرار تطور الصناعة الصيدلانية، سيظل QbD جانبًا أساسيًا في تطوير الأدوية وتصنيعها، ويهدف هذا المقال إلى توفير فهم شامل لمنهج QbD للعاملين في مجال الصيدلة.

الكلمات الدالة: التصميم بالجودة، تطوير الأدوية، الأشكال الصيدلانية الصلبة، تصنيع الأدوية، النهج القائم على المخاطر.

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Comparison of Job Satisfaction by Alumni and Student Medical Representatives and The Associated Factors in Iraq

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ABSTRACT

Background: Job satisfaction is a multifaceted construct that involves the intricate interplay of an employee's emotional, cognitive, and behavioral characteristics with their job.

Objectives: To explore the difference in job satisfaction among alumni and student medical representatives (MRs), as well as to identify the factors that influence the job satisfaction of MRs.

Methods: A cross-sectional face-to-face survey was conducted with medical school students and alumni working as MRs in various Iraqi universities.

Results: A total of 449 MRs participated in this study. A statistically significant difference was found in job satisfaction items, namely recognition, responsibility, salary, and working conditions between alumni and student MRs, with higher values observed in the alumni MRs group (p-values of 0.008, 0.003, 0.029, and 0.025, respectively). More than half of the participants had low levels of job satisfaction.

Conclusions: Alumni and student MRs have similar levels of job satisfaction. The factors that significantly contribute to job satisfaction among alumni MRs include recognition of good performance, increased autonomy and responsibilities, competitive salaries, and improved working conditions within the company. The satisfaction of MRs is significantly influenced by the quality of products and the reputation of the company.

Keywords: Satisfaction, Alumni, Student.

Introduction

The pharmaceutical market in Iraq exhibits distinct characteristics that set it apart from other nations. Despite a substantial gross domestic product that surpassed $110 billion in 2019, only $5 billion were designated for the national health sector, with only a quarter of this budget being utilized for the reimbursement of medicines [1]. The shortage of supplied medicines in public healthcare institutions has created an opportunity for the private sector to step in and address the gap through international companies and their local distributors [2]. The most recent records from the Iraqi Ministry of Health indicate that the pharmaceutical market in Iraq is highly competitive, with over 8,600 registered trade drugs and 28 national pharmaceutical companies [3]. To expand their market share or penetrate new markets, pharmaceutical companies often employ medical representatives (MRs) as a strategic approach [4].

The role of a medical representative (MR) involves representing a pharmaceutical manufacturer or distributor by providing information and persuading healthcare professionals about the quality, effectiveness, and safety of medicinal drugs. The MR's objective is to encourage the prescribing, purchase, and/or use of these drugs [5, 6]. The profession of a medical representative was initially

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documented in the early 1900s in Europe [6]. This profession has implemented various strategies to enhance outcomes by targeting prescribers through calls, visits, conferences, and symposia, as well as customers or end-users through advertising using brochures and media. Additionally, the MRs profession recruit researchers to further improve their techniques [7]. According to Buckley's (2004) study, the majority of MRs and marketing campaigns are targeted toward physicians. This is achieved through various means, such as in-person visits accompanied by the distribution of free medical samples, brochures, and the provision of current research that supports the efficacy of their products [8, 9].

Job satisfaction is a multifaceted construct that involves the intricate interplay of an employee's emotional, cognitive, and behavioral characteristics with their job [10, 11]. It describes the extent of comfort and contentment of an employee during work [12]. Recently, organizations and pharmaceutical companies have directed their efforts toward exploring and enhancing employees' job satisfaction, with the ultimate goal of improving organizational productivity [10]. Studies by Bruke (2005) and Ahmed and Rafiq (2013) revealed that pharmaceutical companies with high levels of employee satisfaction are more competitive and profitable than their counterparts [13, 14]. The company's strategy of incentivizing, rewarding, and providing professional leadership to its employees is aimed at ensuring that individuals meet management's expectations and deliver good results [14]. Despite the significance of job satisfaction among medical representatives (MRs), a review of the literature has revealed a paucity of research investigating this topic. This study aims to explore the difference in job satisfaction among alumni and student MRs, as well as to identify the factors that influence the job satisfaction of MRs.

METHODS

A cross-sectional face-to-face survey was conducted between October 1st and November 15th, 2022. The study received ethical approval from the research committee at the College of Pharmacy, Mosul University. The participants were current students of all academic levels or graduates from Iraqi medical schools with different specialties, including Pharmacy, Medicine, Dentistry, Nursing, and Veterinary medicine, who are currently full-time or part-time working or have previously worked as medical representatives in pharmaceutical companies for marketing medicines. The data collection process was conducted at various locations, including private community pharmacies, drug stores, and academic institutions such as colleges of pharmacy, medicine, and dentistry at the universities of Mosul, Baghdad, Duhok, Hawler, Kirkuk, Najaf, and Basrah. The interviews were conducted by IAJ, TAT, FFA, ZSS, and TMJ in a location convenient for the participants. The interviews took an average of 10 minutes to complete. Before conducting the interviews, the interviewers described the aims of the study and ensured that the interviews would be solely intended for scientific research. The researchers provided reassurance to the participants that the survey questionnaire would not gather any personally identifying information. Additionally, they emphasized that each interviewee's response would be anonymized, thereby ensuring that the respondent's identity remains confidential and cannot be tracked. Before commencing the interview, the interviewee provided informed consent to participate in the study, and they were also informed of their right to withdraw from the study at any time. After conducting the analysis, the gathered data were securely stored in a locked cabinet located within the chief investigator’s office at the College of Pharmacy.

Study instrument

The study questionnaire comprised three sections. The first section aimed to explore demographic characteristics, including gender, age, marital status, province of employment, educational background, employment status, type of company, years of work experience, job position, work zone, and salary. The second section of the study

comprised a validated job satisfaction scale. The scale consisted of questions pertaining to various aspects such as achievement, recognition, responsibility, opportunity for advancement, company policy and administration, supervision, salary, interpersonal relations, and working conditions. This scale was obtained from Roopai's (2012) study, which utilized content and criterion validity measures to validate the scale [15]. The job satisfaction scale for each item ranged from 1 to 5, with 1 indicating a high level of dissatisfaction (very unsatisfied) and 5 indicating a high level of satisfaction (very satisfied). The overall score exhibited a range of 5 to 45. The assessment of job satisfaction factors was conducted using a five-point Likert scale, ranging from -2 to 2, where -2 indicated a factor of very low importance, -1 indicated a factor of low importance, 0 indicated a neutral factor, 1 indicated an important factor, and 2 indicated a very important factor.

The final section of the study aimed to explore the factors that influenced the satisfaction of the participants. These factors include training programs, the attitudes of the physician, pharmacist, supervisor, and physician gatekeeper, sales target, location of work, working hours, quality of the products, and the reputation of the company. This section was based on a separate study [16]. The questionnaire's validity was assessed through face and content validity, whereby it underwent review by a panel of 11 clinical pharmacy experts from the University of Mosul and Nineveh University, who were selected based on their academic credentials. The panel did not make any modifications to the questionnaire. Subsequently, the questionnaire underwent a pilot study with a cohort of 20 participants. The reliability of the questionnaire was confirmed, with the Cronbach's α coefficient being 0.733. The participants in this pilot study were pharmaceutical medical representatives employed by various pharmaceutical companies operating within Nineveh province. The participants in the pilot study were excluded from the main study.

Statistical analysis

Descriptive and inferential statistical analyses were conducted using the Statistical Package for Social Science (SPSS) version 25. The determination of the cut-off point for the satisfaction level was achieved through the utilization of the median split method [17]. The Kolmogorov-Smirnov test results indicated that the data were non-normally distributed. Nonparametric statistical tests, including Spearman correlation, Mann-Whitney U test, and Kruskal-Wallis test, were utilized to assess the correlation between individual satisfaction items and the overall level of job satisfaction, as well as to identify any differences in mean scores. The study findings are reported using numerical values and percentages for categorical data, while normally distributed quantitative data are presented as means with standard deviation (SD), and non-normally distributed quantitative data are presented as median with interquartile range (IQR). The determination of the key factors that impact the level of satisfaction within each group was achieved through the computation of the total points assigned to each factor based on the responses received.

RESULTS

The study involved the participation of 449 individuals identified as MRs, including both alumni MRs (256) and student MRs (193). The majority of the participants were male, single, and residing in the mid-north region of Iraq. Over fifty percent of the participants in the study had either graduated or were currently studying at the College of Pharmacy. Additionally, they were employed as Medical Representatives (MRs) in generic companies, with a work location limited to the city center, and earning a monthly salary exceeding $600 (Table 1).

Table 1: Demographic characteristics of participating MRs

Variables		N	Alumni MRs (N=256)	Student MRs (N=193)
Age ǂ		449	27 (25-30)	23 (22-24)
Gender	Male	347	216 (84.4%)	131 (67.9%)
	Female	102	40 (15.6%)	62 (32.1%)
Marital state*	Single	302	129 (50.4%)	173 (89.6%)
	Married	143	125 (48.8%)	18 (9.3%)
	Divorced	2	1 (0.4%)	1 (0.5%)
	Widow	2	1 (0.4%)	1 0.5%)
Provinces*	Kurdistan	30	21 (8.2%)	9 (4.7%)
	Mid-north	352	200 (78.1%)	152 (78.8%)
	South	67	35 (13.7%)	32 (16.6%)
College of study or graduation*	Pharmacy	246	130 (50.8%)	116 (60.1%)
	Medicine	59	31 (12.1)	28 (14.5%)
	Dentistry	41	19 (7.4)	22 (11.4%)
	Others	103	76 (29.7%)	27 (14%)
Working as a med rep*	ex-rep	175	97 (37.9%)	78 (40.4%)
	med rep	274	159 (62.1%)	115 (59.6%)
Company type*	Brand	109	74 (28.9%)	35 (18.1%)
	Generic	256	154 (60.2%)	102 (52.8%)
	I do not know	84	28 (10.9%)	56 (29.0%)
Years of working ǂ		449	3 (1-4)	1 (1-2)
Position inside the company*	Owner	6	3 (1.2%)	5 (2.6%)
	Team leader	47	30 (11.7%)	17 (8.8%)
	Marketing manger	7	6 (2.3%)	0 (0 %)
	Executive manager	3	2 (0.8%)	0 (0 %)
	Supervisor	37	35 (13.7%)	2 (1.0%)
	Medical representative	261	133 (52%)	128 (66.4%)
	Sale man	88	47 (18.4%)	41 (21.2%)
Work zone*	The city center only	282	161 (62.9%)	121 (62.7%)
	The city borders only	30	10 (3.9%)	20 (10.4%)
	The city center and borders	51	30 (11.7%)	21 (10.9%)
	The whole province	86	55 (21.5%)	31 (16.1%)
Salary*	Less than 200$	28	10 (3.9%)	18 (9.3%)
	Between 200 and 400	67	29 (11.3%)	38 (19.7%)
	Between 400 and 600	91	46 (18.0%)	45 (23.3%)
	More than 600	263	171 (66.8%)	92 (47.7%)

ǂ presented as median ± interquartile range; * presented as frequency and percentage

Job satisfaction items

With respect to the items contributing to job satisfaction among alumni and student MRs, the results show that interpersonal relations were ranked as the most highly scored item by the alumni MRs. The Mann-Whitney U test results indicate a statistically significant difference in recognition, responsibility, salary, and working conditions between alumni and student MRs, with higher values observed in the alumni MRs group (p-values of 0.008, 0.003, 0.029, and 0.025, respectively) (Table 2).

Table 2 Alumni and student MRs job satisfaction items

Job satisfaction items	Alumni MRs (N=256) Median (IQR)	Student MRs (N=193) Median (IQR)	*z-value*	*p-value*
Achievement	3 (3-5)	3 (3-5)	-1.754	0.079
Recognition	3(3-5)	3 (1-3)	-2.632	0.008*
Responsibility	3 (3-5)	3 (1-3)	-2.986	0.003*
Opportunity for advancement	3 (1-4)	3 (1-3)	-1.551	0.121
Company policy & administration	3 (3-5)	3 (3-4)	-0.920	0.357
Supervision	3 (3-5)	3 (3-5)	-1.025	0.305
Salary	3 (3-5)	3 (1-5)	-2.185	0.029*
Interpersonal relations	5(3-5)	5 (3-5)	-1.838	0.066
Working conditions	3 (1-4)	3 (1-3)	-2.238	0.025*

Satisfaction level

The findings illustrated in Table 3 indicate that the job satisfaction levels between the two groups are comparable, with a majority of participants - encompassing both alumni and student Medical Representatives (MRs) - reporting low levels of job satisfaction.

Table 3 Satisfaction levels of alumni and student MRs

Level of satisfaction	Alumni MRs	Student MRs
Low satisfaction	136 (53.1%)	99 (51.3%)
High satisfaction	120 (46.9%)	94 (48.7%)

The correlation between the overall satisfaction scores and demographic characteristics of alumni and student MRs

The Spearman correlation analysis results show positive correlations between the overall satisfaction score and the duration of work experience in both alumni and student MRs. The p-values for these correlations were 0.007 and 0.025, respectively (Table 4).

Table 4 correlation between overall satisfaction level and demographic characteristics of alumni and student MRs

Variables	Alumni MRs (N=256)		Student MRs (N=193)	
	Rho	*p*-value	rho	*p*-value
Age	0.078	0.211	0.002	0.974
Number of years of work	0.168	0.007	0.14	0.025

rho: Spearman's correlation

The difference between the overall satisfaction scores and demographic characteristics of alumni and student MRs

Regarding alumni MRs, the Kruskal-Wallis test results reveal statistically significant differences in the overall satisfaction level among groups with different employment statuses, specifically between those currently working as MRs and those who have resigned. Additionally, the results show significant disparities in satisfaction levels among MRs employed by different types of companies. Conversely, in the case of student MRs, the Kruskal-Wallis test results indicate statistical differences in satisfaction levels among groups holding diverse positions within the company and earning varying salaries (Table 5).

Table 5 The difference between overall satisfaction level and demographic characteristics of alumni and student MRs

		Alumni MRs		Student MRs	
Demographics variable		**Mean (SD)**	***p*-value**	**Mean (SD)**	***p*-value**
Gender*	Male	28.42 (8.84)	0.623	27.48 (8.84)	0.813
	Female	27.9 (7.89)		27.29 (7.40)	
Marital status**	Single	27.66 (8.39)	0.150	27.37 (8.28)	0.862
	married	29.20 (8.84)		27.61 (9.93)	
	divorced	27 (0)		30 (0)	
	Widow	9 (0)		30 (0)	
Province**	Kurdistan	29 (6.87)	0.45	23.77 (8.21)	0.189
	mid-north	28.69 (8.92)		27.92 (8.35)	
	South	25.94 (8.09)		26.06 (8.42)	
Profession **	pharmacist	28.28 (9.18)	0.424	27.38 (8.22)	0.545
	physician	27.61 (8.7)		27.67 (7.01)	
	Dentist	25.73 (8.98)		25 (8.47)	
	Others	29.38 (7.66)		29.29 (10.12)	
Have you worked as a med rep*	Ex-rep	26.69 (8.72)	0.024	26.05 (8.57)	0.064
	med rep	29.34 (8.54)		28.35 (8.15)	
Company type**	Brand	32.29 (7.65)	0.001	27.88 (8.96)	0.322
	generic	26.98 (8.57)		28.09 (8.28)	
	I do not know	25.32 (8.67)		25.91 (8.15)	
Working zone **	city center only	27.70 (8.55)	0.338	27.04 (8.38)	0.418
	whole province	29.47 (9.0)		26.74 (9.05)	
	whole city	29.63 (9.98)		28.85 (8.03)	
	city borders	28.5 (3.1)		29.25 (7.84)	
Position inside the company**	Owner	35.33 (9.6)	0.184	22.2 (7.79)	0.002
	team leader	30.63 (9.31)		32.76 (7.15)	
	marketing manager	31.5 (5.75)		11(1.14)	
	executive manager	32 (1.41)		12 (1.4)	
	supervisor	30.42 (8.52)		23 (2.82)	
	med rep	27.12 (8.46)		26.15 (7.8)	
	sale man	27.74 (8.96)		30.02 (9.44)	
Salary **	less than 200$	24 (9.67)	0.13	24.66 (9.97)	0.013
	200$- 400$	28.03 (8.01)		28.33 (7.87)	
	400$ - 600$	25.93 (9.44)		25.06 (7.83)	
	more than 600$	29.29 (8.39)		29.31 (8.81)	

* Mann Whitney; ** Kruskal-Wallis test

Factors affecting alumni and student MRs job satisfaction

Alumni Medical Representatives (MRs) ranked factors that positively influence or predict job satisfaction in the following descending order: quality of products, company reputation, attitude of the pharmacist, working hours, sales target, work location, training programs, attitude of other MRs, and physician's attitude. The participants highlighted the negative impact of the gatekeeping attitude of physicians on their job satisfaction. Conversely, student MRs rated the factors that positively influence or predict job satisfaction slightly differently from the alumni MRs. In order of importance, as determined by the ratings, they were: quality of products, working hours, pharmacist's attitude, company reputation, work location, supervisor's attitude, sales target, training programs, and physician's attitude (Figure 1).

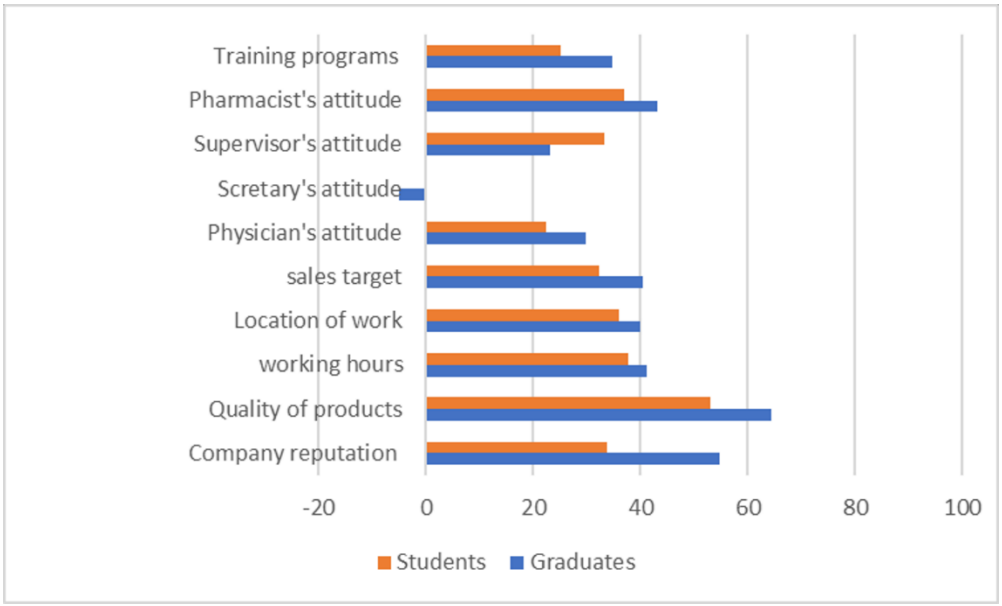

Figure 1 Factors affecting alumni and student MRs job satisfaction

MRs plans to change job

The participating MRs were asked about their preferences to either remain with their current company, transition to a different organization, or leave the MR profession entirely. The results of the survey indicate that a majority of alumni MRs, specifically 57%, expressed their intention to remain employed with their current company. On the other hand, over one-third of the respondents expressed their desire to permanently leave the MR profession. In contrast, a smaller proportion of student MRs reported their intention to remain employed with their current company (Table 6).

Table 6 the plans of alumni and students MRs to change job

Plans to change job	Alumni MRs	Student MRs
Leave MR job forever	98 (38.3%)	66 (34.2%)
Stay with the same company	146 (57%)	81 (42.0%)
Move to another company	12 (4.7%)	46 (23.8%)

DISCUSSION

The present study investigates the difference in job satisfaction between alumni and student MRs, as well as the various factors that contribute to job satisfaction. This study intended to enhance the job satisfaction of medical representatives and address the knowledge gap in this area. Previous research in this field has primarily focused on alumni, thus highlighting the need for further investigation of the experiences of current medical representatives. In order to comprehend the results of the comparison between alumni and student MRs, it is crucial to consider that working students may differ significantly from working alumni in various aspects. For instance, their financial motivation and propensity to leave a job may be unpredictable [18].

Job satisfaction is a multidimensional concept. Previous research has indicated that these dimensions are influenced by a combination of demographic and work-related factors [19–21]. Herzberg's Theory posits that job satisfaction is influenced by both intrinsic factors, such as achievement, recognition, responsibility, and opportunities for advancement, as well as extrinsic factors, including company policy and administration, supervision, salary, interpersonal relationships, and working conditions [22, 23]. The findings of the present study suggest that alumni MRs consider interpersonal relations to be a crucial and foundational aspect of their job satisfaction. The quality of social life and productivity of the MR can be influenced by the interpersonal relationships they establish with their manager, peers, colleagues, and external contacts within and outside the company. According to a study by Kreitner et al. (2002), positive interpersonal relationships can serve as a motivator and enhance job satisfaction [24]. The alumni MRs' ratings of interpersonal relations were higher than those of the student MRs, as the former recognized the importance of building relationships over time and through effective communication skills. Moreover, a considerable number of medical institutions incorporate communication skills training into their academic program [25, 26].

The findings of the present study indicate that the recognition item holds greater significance for alumni MRs compared to student MRs. Recognition refers to acknowledging or commending a task that has been accomplished, and to differentiate between scenarios where tangible incentives are provided in conjunction with acknowledgments of achievement and those where such incentives are absent. The studies conducted by Bodla and Naeem (2004) and Malik and Naeem (2009) have demonstrated that recognition plays a crucial role as a motivational factor for MRs [27, 28]. This finding is consistent with the research conducted by Roopai (2012) [15].

The item of responsibility held greater significance for alumni MRs in comparison to student MRs. Responsibility, in this context, refers to the act of being entrusted with tangible duties and possessing the necessary authority to execute them proficiently. According to a study conducted by Graham and Messner (1998), there is a positive correlation between job responsibilities and job satisfaction among employers [29]. The greater emphasis on responsibility exhibited by alumni may also be attributed to their proclivity for working independently to enhance their sales targets and profits, as well as their potential for career advancement.

The salary was identified as a significant item in job satisfaction among alumni MRs, as opposed to students' MRs. As alumni rely on their salary as their primary source of income, while students typically work to supplement their finances and gain practical experience. According to a study conducted by Kabir and Parvin (2011), salary was identified as the most significant motivational factor for job satisfaction among employees working in pharmaceutical companies [30].

The alumni MRs rated the quality of working conditions as one of the most significant items influencing job satisfaction, as opposed to the student MRs. Robbins (2001) study suggested that the level of job satisfaction

experienced by employees can be influenced by their working conditions, as employees prioritize a comfortable physical work environment. This, in turn, can lead to a higher level of job satisfaction. Employees may perceive that unfavorable working conditions can result in poor performance, given the mental and physical demands of their jobs [31]. Student MRs might be less affected by the working conditions since they are working in part-time jobs and/or due to the fewer chances that are available for students. Pharmaceutical firms prefer to hire alumni rather than students. The impact of working conditions on students' MRs' job satisfaction may be explained by their part-time employment status and limited job opportunities. Pharmaceutical companies exhibit a preference for recruiting alumni over current students.

The results of the present study indicated that the majority of Iraqi MRs exhibit a low level of job satisfaction. The findings indicate that alumni and student MRs exhibit comparable levels of job satisfaction. The present study findings are consistent with those of Saleh (2018), who reported that 73% of Ethiopian medical representatives exhibited low levels of job satisfaction [32]. However, the findings of this study were inconsistent with those of Roopai's (2012) study, which reported that 62% of South African medical representatives had high levels of job satisfaction [15].

The results of the present study indicated that there was a positive correlation between the number of years of work and the overall level of satisfaction in both groups. The level of satisfaction among MRs increases with their accumulated experience. According to Jaffar et al (2017) research, there is a positive correlation between the length of service of MRs within a company and their job satisfaction [33]. In contrast, a study conducted in India discovered that the job satisfaction of MRs was not significantly associated with their length of service within a company. This finding can be attributed to the tendency of MRs to leave their jobs after a few years in India [15].

The present study found that job satisfaction varied between current and former MRs, with higher levels of satisfaction reported among those who are currently employed in the field. This phenomenon is not unexpected, as the persistence of their employment suggests a level of contentment with their work.

The results of the present study also indicated that the level of job satisfaction varied between MRs employed in brand pharmaceutical companies and those employed in generic companies, and those who were uncertain about their company's drug production status. A greater level of satisfaction was observed among alumni MRs who were employed in brand companies. The present study findings are consistent with those of Arafat et al. (2015), who reported that job satisfaction among Bangladeshi MRs was higher among those employed in brand companies [34]. Similarly, the job satisfaction level was higher among student MRs who worked as team leaders within the company. According to Bakhuys Roozeboom et al. (2020) study, individuals occupying higher positions within their organizations experienced lower levels of work stress and organizational stress, leading to higher levels of job satisfaction [35]. The level of job satisfaction experienced by MRs was found to be positively correlated with their salary, with those earning a higher salary reporting greater job satisfaction than those with a lower salary. The study conducted by Jaffar et al. (2017) demonstrated a positive correlation between the job satisfaction of MRs and their promotion and salary levels [33]. Previous research has indicated a positive correlation between job satisfaction and both higher salaries and improved job positions within the workplace [15, 36].

The present study examined the factors that impact the job satisfaction of MRs. This study found that the quality of the products promoted by MRs and their ability to persuade healthcare professionals to prescribe or sell these products were identified as the most significant positive factors by both groups. The alumni MRs have conveyed that the reputation of the drug manufacturing company is the second most significant factor that contributes to their

satisfaction, whereas this factor holds less importance for the student MRs. This finding is consistent with the research conducted by Musleh and Al-Dmour (2011) [37]. This could be attributed to the eagerness of student MRs to secure employment at any company, coupled with the limited job offers available to them. In contrast, alumni MRs have access to a wider range of job opportunities, which allows them to be more selective and opt for companies with a better reputation.

The pharmacist's attitude was identified as the most significant factor that positively influenced the satisfaction of both groups. This could be ascribed to the greater proportion of pharmacists and/or pharmacy students who took part in the study. Furthermore, the potential good relationship between pharmacists and MRs, as pharmacies serve as the ultimate destination for medical promotion efforts. The attitude of supervisors was found to be more significant for students than for alumni MRs, given the greater need for training among students to achieve professional readiness.

Conversely, the attitude of physicians' gatekeepers towards medical representatives has been identified as a detrimental factor that impacts the job satisfaction of alumni MRs. As physicians' gatekeepers, these individuals have the authority to regulate the access of MRs to healthcare professionals, which may impede the representatives' ability to conduct medical representation calls. The student MR's level of enthusiasm and persistence may potentially mitigate the impact of this factor.

Despite the comparatively lower levels of job satisfaction reported by both alumni and student MRs, a greater proportion of alumni MRs indicated a desire to remain with their current company. A smaller proportion (42%) of student MRs indicated their intention to remain employed with their current company. This finding may be attributed to the older employees' preference for job stability compared to younger ones. Additionally, this could be attributed to the comparatively lower salaries provided to MRs who are still students, in contrast to those who have graduated, as well as the necessity for them to discontinue their work at some point to fulfill their education.

The present study bears a number of strengths and limitations. This research is pioneering in its exploration, comparison, and contrast of job satisfaction levels among alumni and student Medical Representatives (MRs) in Iraq and the Middle East region. It is particularly significant due to its focus on the distinctive opportunity offered by Iraqi pharmaceutical companies, which employ students as MRs. However, the study also carries certain limitations that may impact its findings' generalizability. Nevertheless, eliminating these limitations posed a significant challenge. (1) The subjective nature of job satisfaction, which is influenced by psychographic factors, can result in varying levels of satisfaction among MRs from the same company who are working under similar conditions. (2) The Iraqi market is characterized by a large number of pharmaceutical companies that are dispersed across various locations and are subject to different regulatory frameworks. (3) The dynamic nature of the profession of MRs poses a challenge due to the limited availability of interview opportunities. (4) The study was designed to incorporate interviews with MRs from various provinces in Iraq. The combination of the aforementioned reasons, coupled with the limitations of time, led to the selection of convenient sampling as the most appropriate method for this study. Furthermore, we did not conduct regression analysis to build a model with the variables which are significantly and independently associated with the study outcome. Given the aforementioned limitations, it is recommended that a more extensive survey be conducted to encompass all pharmaceutical companies operating in Iraq. This will provide a more comprehensive understanding of the satisfaction levels among this population.

CONCLUSIONS

The current study reveals a similarity in job satisfaction levels between alumni and student Medical Representatives (MRs). Significant contributing factors to job satisfaction among the alumni MRs included

recognition of good performance, increased autonomy and responsibilities, competitive salaries, and enhanced working conditions within the company. Alumni MRs working in brand companies displayed higher satisfaction. Meanwhile, holding higher positions within the company and earning higher salaries were associated with greater satisfaction among student MRs. Both groups were significantly influenced by the quality of products and the company's reputation, highlighting these factors as being of paramount importance. Policymakers and managers of pharmaceutical companies should give greater consideration to these determinants in order to improve employee satisfaction.

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مقارنة الرضا الوظيفي من قبل الخريجين والطلاب المندوبين الطبيين والعوامل المرتبطة بها في العراق

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ملخص

الخلفية: الرضا الوظيفي هو تفاعل متعدد الأوجه للخصائص العاطفية والمعرفية والسلوكية للموظف مع وظيفته.
الأهداف: استكشاف الاختلاف في الرضا الوظيفي بين المندوبين الطبيين من الخريجين والطلاب وكذلك تحديد العوامل التي تؤثر على الرضا الوظيفي للمندوبين الطبيين.
الأساليب: تم إجراء مسح مقطعي مستعرض وجهًا لوجه مع طلاب وخريجي كليات المجموعة الطبية في العديد من الجامعات العراقية.
النتائج: شارك 449 مندوبا طبيا في هذه الدراسة. فروق ذات دلالة إحصائية في عناصر الرضا الوظيفي وهي؛ الاعتراف والمسؤولية والراتب وظروف العمل بين المندوبين الطبيين من الخريجين والطلاب، مع وجود قيم أعلى لوحظت في مجموعة الخريجين (قيم p 0.008، 0.003، 0.029، و 0.025، على التوالي). أكثر من نصف المشاركين لديهم مستويات منخفضة من الرضا الوظيفي.
الاستنتاجات: لدى الخريجين والطلاب مستويات مماثلة من الرضا الوظيفي كانت العوامل التي ساهمت بشكل كبير في الرضا الوظيفي بين الخريجين هي الاعتراف بالأداء الجيد، وزيادة الاستقلالية والمسؤوليات، والرواتب التنافسية، وتحسين ظروف العمل داخل الشركة. يتأثر رضا المندوبين الطبيين بشكل كبير بجودة المنتجات وسمعة الشركة.

الكلمات الدالة: رضا، خريج، طالب.

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Methanol Leaves Extract of *Zingiber officinale* (Roscoe) exhibited Anti-Obesity Effect in Wistar Rats Fed with a High Fat Diet

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ABSTRACT

This study evaluated the anti-obesity properties of the methanol extract of *Zingiber officinale* leaves in Wistar rats. Thirty male rats were distributed into five groups, with six rats in each group, and different groups were treated with a normal fat diet (NFD), high-fat diet (HFD), HFD + orlistat (20 mg/kg) p.o, HFD + *Zingiber officinale* (200 mg/kg) p.o, and HFD + *Zingiber officinale* (400 mg/kg) p.o for fifty-six days. After all administrations, the animals were sacrificed by cervical dislocation, and various biochemical analyses were carried out. Results showed that there was a significant decrease ($p < 0.05$) in body weight and adiposity in the *Zingiber officinale*, NFD, and orlistat groups compared to the HFD control. However, there was no significant difference in the body weights of rats in the *Zingiber officinale* groups compared to the NFD control and orlistat groups. Furthermore, rats in the *Zingiber officinale* groups had normal lipid concentrations, antioxidant status, adipokines, cytokines, liver, kidney, and cardiac function parameters that were comparable to orlistat and normal control but in contrast with the HFD control. Findings from the study suggest that *Zingiber officinale* leaves have significant anti-obesity, antioxidant, and anti-inflammatory properties.

Keywords: *Zingiber officinale*, body weight, adipose tissues, cytokines, orlistat, adipokines, antioxidants.

1. INTRODUCTION

Obesity is a chronic disease involving an excess amount of body fat that develops as a result of a long-term energy imbalance, i.e., excessive caloric consumption and insufficient energy output[1]. Research has demonstrated that obesity decreases life expectancy because it raises the possibility of developing numerous medical complications, such as type 2 diabetes mellitus, dyslipidemia, cardiovascular diseases, and some types of cancer[2].

Orlistat, lorcaserin, and a combination of phentermine and topiramate are available for the management of obesity. However, they may cause gastrointestinal, kidney, and heart problems[3,4]. Presently, no drug provides continuous and reasonable weight loss with few side effects[5]. Therefore, various efforts are made to lower weight with pharmacological agents from plants that may bring minimal adverse reactions[6]. Traditionally, plants have been utilized as medicines for treating various diseases. Leaves of *Cymbopogon citratus* are used in the treatment of cold and flu[7, 8]. *Moringa oleifera* (leaves and seeds), *Momordica charantia* and *Tinospora crispa* leaves are used in the treatment of diabetes[8,9,10]. It has been reported that diarrhea is treated with *Psidium guajava* leaves[11], while indigestion and stomachache are treated with leaves of *Capraria biflora*[8]. Fruits of *Passiflora edulis* are used in the treatment of hypertension[11]. *A.*

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falcata and *M. aurea* leaves are used in the management of inflammation and skin irritations[12]. Research has revealed that various herbal plants have anti-obesity properties. *Curcuma longa* rhizomes have been found to reduce body weight, serum lipids, and suppress adipocyte differentiation in high-fat diet-induced rats[13]. *In vitro* study by Kim *et al.* [14] showed that a mixture of peels of *Citrus unshiu* and Diospyros kaki fruits has pancreatic lipase inhibition activity. Prunus salicina extract attenuated adipogenesis in murine 3T3-L1 adipocytes cells[15]. Findings made by Maia-Landim *et al.*[16] showed that *Garnicia cambogia* and glucomannan from Amorphophallus konjac reduced appetite and weight in obese people while findings by Nepali *et al.* [17] showed that *Chrysanthemum indicum* inhibited adipogenesis in high fat diet induced mice.

Zingiber officinale, commonly called 'ginger,' is a very important medicinal plant. Traditionally, ginger rhizomes are often used to cure many illnesses, such as indigestion, loss of appetite, flatulence, nausea, vomiting, allergic reactions, acute and chronic cough, common cold, fever, allergic rhinitis, sinusitis, bronchitis, respiratory troubles, headache, backache, and toothache[18]. It is also used for the treatment of primary dysmenorrhea[19]. Ginger leaves have been employed as a flavor for foods in Asian traditional medicine. They have been used to reduce toothache, promote digestion, and reduce constipation[20]. Reports have shown that ginger rhizomes possess hypoglycemic activity and ameliorate type I diabetes [21]. Al-Amin *et al.* [22] also demonstrated that ginger rhizomes possess hypolipidemic activity. Research has shown that extract of ginger rhizomes possess weight lowering, renoprotective, and antioxidant properties[23]. Ginger leaves extract have been reported to possess antioxidant activities and phytochemicals including flavonoids, tannins, saponins, and glycosides [24].

There is a paucity of information on the anti-obesity effect of *Zingiber officinale* leaves, and most studies on the plant are on the rhizomes. Therefore, this study aimed at evaluating the anti-obesity property of *Zingiber officinale* leaves in Wistar rats fed with a high-fat diet.

2. MATERIALS AND METHODS

2.1 Plants

Fresh leaves of *Zingiber officinale* were harvested from a private farm in Ondo City, Ondo State, Nigeria, and authenticated by a Botanist at the University of Benin. A specimen with voucher number UBHz 368 was deposited at the herbarium. The leaves of *Zingiber officinale* (700g) were dried in the air, pulverized, and immersed in absolute methanol (7.5mL) for 72 hours. Filtration was thereafter carried out using chiffon filter. A rotary evaporator was then used to concentrate the filtrate (temperature, 40 oC), and the resulting paste was further dried in an incubator at 40oC and stored in an airtight sterile bottle. The percentage yield of the extract was 4.8%.

2.2 Experimental animals

Wistar rats (male) with weights ranging from 130g to 150g were obtained from the animal house of the University of Medical Sciences (UNIMED), Ondo, Nigeria. Ethical approval for the use of animals was obtained from the UNIMED Research Ethics Committee with the number UNIMED-AREC/Apv/2022/015

The animals were housed in clean cages at room temperature (25 ± 1oC), with a 12-hour light/dark cycle, and the litter was changed every day. The experiments were carried out in compliance with internationally accepted principles for the use and care of laboratory animals[25] .

2.3 Experimental procedure

Obesity was induced by a high-fat diet (HFD). The diets were formulated based on the method of Cha and Jones[26]. The composition of the Normal Fat Diet (NFD) and HFD is shown in Table 1. Thirty Wistar rats were distributed into 5 groups, each comprising 6 rats. The dried extract of *Zingiber officinale* leaves was dissolved in distilled water (vehicle) according to the corresponding dose. The rats were subjected to the following for 56 days:

- Group 1: NFD + distilled water (NFD control)
- Group 2: HFD + distilled water (HFD control)
- Group 3: HFD + Orlistat (Reference anti-obesity drug; 20mg/kg, *p.o*
- Group 4: HFD + *Zingiber officinale* (200mg/kg, *p.o*)
- Group 5: HFD + *Zingiber officinale* (400mg/kg, *p.o*)

Table 1: Content of NFD versus HFD

NUTRIENT	NFD(w/w)	HFD(w/w)
Soya beans	15%	15%
Corn starch	60%	40%
Sucrose	10%	10%
Soybean oil	4%	4%
Beef fat	-	20%
Vitamins mix	3.5%	3.5%
Minerals mix	1%	1%
Cellulose	6.5%	6.5%

Weights of rats were taken weekly and feed consumption was recorded daily for eight weeks (56 days).

On the 57th day, the rats were then sacrificed by cervical dislocation. The body length (nose-to-anus) was measured, and their values were used to calculate the following anthropometric parameters:

Body mass index (BMI): body weight (g)/length^2 (cm^2)[27].

Lee's index: Body weight (g) (cube root) /nose-to-anus length (cm) [27].

Epididymal fat, retroperitoneal fat were collected and weighed. Adiposity index was calculated with the formula of Boustany *et al.*[28] as follows:

$$Adiposity\ index = \frac{\text{Epididymal fat} + \text{Retroperitoneal fat}}{\text{Weight of body (g)} - (\text{Epididymal fat} + \text{Retroperitoneal fat})} \times 100$$

Blood samples were collected via cardiac puncture into sterile tubes and allowed to stand for 30 minutes at 20-25^{o}C. The clear serum was separated at 2500*g* for 15 minutes using a centrifuge.

Liver and heart samples were harvested. A sample of the liver (1g) was homogenized in 9 mL of buffer (sodium phosphate) pH 7.0, while 0.5 g of the heart was homogenized in 4.5 mL of buffer at pH 7.0. The homogenates were centrifuged at 1000 g for 15 minutes, and the supernatant was stored for subsequent analyses.

1.4 Serum liver function tests

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, albumin, and total protein were assayed in the serum using kits from Randox Laboratories Ltd, Crumlin, County Antrim, United Kingdom.

1.5 Serum cardiac function tests

Creatine kinase (CK) and lactate dehydrogenase (LDH) were assayed in the serum using kits from Randox Laboratories Ltd, Crumlin, County Antrim, United Kingdom.

2.6 Serum kidney function tests

Sodium, potassium, chloride, and bicarbonate ions were measured with kits from Fortress Diagnostics, Antrim, United Kingdom. Creatinine and urea were measured using kits from Randox Laboratories Ltd, Crumlin, County Antrim, United Kingdom.

2.7 Serum lipid profile assay

Total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and triglycerides (TG) were measured in the serum using kits from Randox

Laboratories Ltd, Crumlin, County Antrim, United Kingdom.

Low-density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C), atherogenic index (AI) and coronary risk index (CRI) were calculated using the formula of Friedewald *et al* .[29] as follows:

LDL-C = TC-(HDL-C +TG/5)

VLDL-C= TG/5

AI = (TC-HDL-C) / HDL-C

CRI = TC / HDL-C

2.8 Serum adipokines and cytokines

Adipokines (leptin and adiponectin) were analyzed in serum using ELISA kits by Elabscience Biotechnology Company, Houston, Texas, USA. Cytokines (tumor necrosis factor alpha (TNFα) and interleukin-6) were analyzed in serum using ELISA kits by Elabscience Biotechnology Company, Houston, Texas, USA.

2.9 Oxidative stress indices

Malondialdehyde (MDA)[30], reduced glutathione (GSH) [31] and glutathione peroxidase (GPx) [32] were assayed in the liver and heart homogenates of rats.

2.10 Histological analyses

The liver and kidney were harvested from the animals and used for histological analyses. They were immersed in 10% phosphate-buffered formalin, cut into tiny pieces, rinsed, and dehydrated in increasing grades of alcohol. The specimens were then cleaned in xylol, embedded in paraffin, sectioned at 4-6 microns thickness, and stained with Hematoxylin and Eosin for histopathological analyses[33].

2.11 Statistical analysis

The results obtained were depicted as mean ± SEM. One-way analysis of variance (ANOVA) was employed to ascertain the difference in mean between the groups. Tukey-Kramer test was utilized to check the significance levels at p-values less than 0.05. Statistical analysis was performed using SPSS version 23.

2. Results

3.1 Anthropometric parameters of rats

3.1.1 Mean body weight

From week 2 to 8, the mean body weight of rats treated with leaves extract of *Z. officinale* was significantly lower ($p < 0.05$) than the HFD control and not significantly different ($p > 0.05$) from the NFD control and orlistat group (Figure 1).

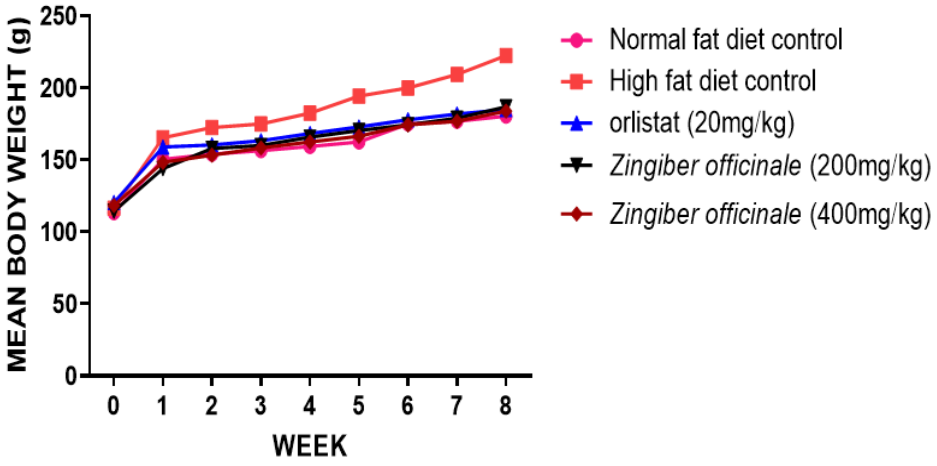

Fig 1: Weekly mean body weight of rats

Results are shown as mean±SEM (n = 6)

3.1.2 Body mass index and Lee's index

Body mass index and Lee's index of rats treated with leaves extract of *Z. officinale* were significantly lower ($p < 0.05$) than the HFD control but were not significantly different ($p > 0.05$) from the normal control and orlistat group (Table 2).

Table 2 Body mass index (BMI) and Lee's index of rats

	BMI (g/cm^2)	Lee's index
NFD control	0.42±0.008^a	0.26 ±0.003^a
HFD control	0.54 ±0.004^b	0.35 ±0.003^b
Orlistat (20mg/kg)	0.47 ±0.003^c	0.28 ±0.002^a
***Z. officinale* (200mg/kg)**	0.43 ±0.003^{ac}	0.26 ±0.003^a
***Z. officinale* (400mg/kg)**	0.43 ±0.004 ^{ac}	0.28 ±0.003^a

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.2 Feed consumption of rats

Daily feed consumed by rats treated with leaves extract of *Z. officinale* was not significantly different *($p > 0.05$)* from, orlistat, NFD control and HFD control (Figure 2).

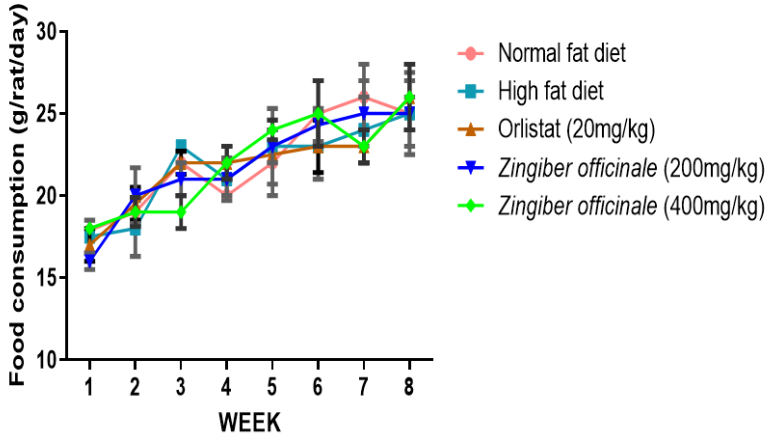

Fig 2: Food consumption of rats

Results are shown as mean±SEM (n = 6)

3.3 Serum lipid profile of rats

The *Zingiber officinale* leaves extract group had significantly lower ($p < 0.05$) total cholesterol, triglycerides, LDLC, VLDLC, AI, and CRI than the HFD control but was not significantly different ($p > 0.05$) from the normal control and orlistat group. However, HDLC was significantly higher ($p < 0.05$) in the *Zingiber officinale* leaves extract group than the HFD control, and no significant difference was seen in HDLC of the leaves extract group compared to the normal control/orlistat group (Tables 3 and 4).

Table 3: Serum lipid profile of rats

	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)
NFD control	79.34 ±1.60[a]	76.50 ± 1.07[a]	29.20 ±1.62[a]	34.74 ±0.22[a]	15.40 ±0.40[a]
HFD control	108.57±1.00[b]	106.30 ±1.08[b]	18.02 ±1.05[b]	69.20 ±0.20[b]	21.25 ±0.10[b]
Orlistat (20mg/kg)	80.00 ± 1.47[a]	86.21 ±0.50[a]	29.00 ±1.20[a]	33.73±1.28[a]	17.25± 0.51[a]
***Z. officinale* (200mg/kg)**	81.40±3.00[a]	75.70 ± 1.50[a]	29.90 ±1.20[a]	36.35 ±1.05[a]	15.15 ±1.22[a]
***Z. officinale* (400mg/kg)**	82.50 ± 1.25[a]	82.25 ± 2.50[a]	28.20 ±1.03[a]	37.07 ±1.08[a]	16.21 ±0.85[a]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

Table 4: Atherogenic index and coronary risk index of rats

	Atherogenic index	Coronary risk index
NFD control	1.72±0.20[a]	2.72±0.82[a]
HFD control	5.05±0.32[b]	6.03±0.50[b]
Orlistat (20mg/kg)	1.76±0.28[a]	2.76±0.78[a]
Z. officinale (200mg/kg)	1.73±0.50[a]	2.70±0.65[a]
Z. officinale (400mg/kg)	1.90±0.25[a]	2.91±0.55[a]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.4 Adiposity of rats

The *Zingiber officinale* leaves extract group had significantly lower ($p < 0.05$) epididymal fat than the HFD control, significantly higher ($p < 0.05$) than the NFD control but was not significantly different ($p > 0.05$) from the orlistat group (Table 5).

The *Zingiber officinale* leaves extract group had significantly lower ($p < 0.05$) retroperitoneal fat and adiposity index than the HFD control but significantly higher ($p < 0.05$) than the NFD control. *Zingiber officinale* (200mg/kg) had significantly lower ($p < 0.05$) retroperitoneal fat and adiposity index than *Zingiber officinale* (400mg/kg) and the orlistat group (Table 5).

Table 5: Epididymal fat, retroperitoneal fat and adiposity index of rats

	Epididymal fat (g)	Retroperitoneal fat (g)	Adiposity index (%)
NFD control	0.73±0.10[a]	0.58±0.01[a]	0.73±0.01[a]
HFD control	3.02±0.20[b]	1.90±0.020[b]	2.26±0.05[b]
Orlistat (20mg/kg)	1.68±0.15[c]	1.35±0.03[c]	1.67±0.01[c]
Z. officinale (200mg/kg)	1.40±0.20[c]	1.25±0.01[d]	1.44±0.01[d]
Z. officinale (400mg/kg)	1.60±0.10[c]	1.40±0.01[c]	1.66±0.01[c]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.5 Kidney function parameters of rats

There was no significant difference ($p > 0.05$) in sodium, potassium, and chloride concentrations of the *Zingiber officinale* leaves extract groups compared to HFD control, orlistat, and NFD control. However, bicarbonate concentration in *Zingiber officinale* leaves extract groups

was significantly higher ($p < 0.05$) than HFD control, orlistat, and NFD control (Table 6).

The concentration of urea in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control but not significantly different ($p > 0.05$) from orlistat and NFD control (Table 7).

The concentration of creatinine in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control but significantly higher ($p < 0.05$) than orlistat and NFD control (Table 7). *Zingiber officinale* (200mg/kg) had significantly lower ($p < 0.05$) creatinine concentration than the *Zingiber officinale* (400mg/kg) group (Table 7).

Table 6: Serum electrolytes concentration of rats

	Na^+(mmol/L)	K^+(mmol/L)	Cl^-(mmol/L)	HCO_3^- (mmol/L)
NFD control	115.17±.148[a]	16.08±0.92[a]	83.45±1.89[a]	142.78±0.94[ac]
HFD control	117.83± 1.60[a]	13.73±1.50[ab]	92.97±4.68[a]	79.00±3.33[b]
Orlistat (20mg/kg)	118.17±2.01[a]	13.89±0.37[ab]	93.03±2.26[a]	135.94±1.19[c]
***Z. officinale* (200mg/kg)**	111.5±1.95[ab]	11.21±0.69[ab]	93.08±2.67[a]	154.39±2.30[d]
***Z. officinale* (400mg/kg)**	117.00±1.68[a]	12.46±0.52[ab]	89.72±2.22[a]	151.38±3.68[d]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

Table 7: Urea and creatinine concentration of rats

	Urea (mg/dL)	Creatinine (mg/dL)
NFD control	27.34±1.31[a]	27.68±1.06[a]
HFD control	38.14±0.35[b]	155.03±1.50[b]
Orlistat (20mg/kg)	26.32±0.42[a]	89.69±1.71[c]
***Z. officinale* (200mg/kg)**	26.23±0.38[a]	124.56±1.72[d]
***Z. officinale* (400mg/kg)**	25.31±0.46[a]	134.41±0.81[e]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.6 Effect of leaf extract of *Zingiber officinale* on liver function parameters of rats

The activity of ALT in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control but significantly higher ($p < 0.05$) than orlistat and NFD control (Table 8).

The activity of AST in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control. *Zingiber officinale* (200mg/kg) had significantly lower ($p < 0.05$) AST activity than *Zingiber officinale* (400mg/kg) and NFD control but was not significantly different from the orlistat group (Table 8).

The activity of ALP in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control. *Zingiber officinale* (200mg/kg) had significantly lower ($p < 0.05$) ALP activity than *Zingiber officinale* (400mg/kg) and orlistat group but was not significantly different from NFD control (Table 8).

The concentration of bilirubin in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than orlistat and HFD control. *Zingiber officinale* (400mg/kg) had significantly lower ($p < 0.05$) bilirubin concentration than *Zingiber officinale* (200mg/kg) and NFD control (Table 9).

The concentration of total protein in *Zingiber officinale* leaves extract groups was significantly greater ($p < 0.05$) than

HFD control but not significantly different from NFD control. *Zingiber officinale* (200mg/kg) had significantly greater ($p < 0.05$) total protein than the orlistat group (Table 9).

The concentration of albumin in *Zingiber officinale* leaves extract groups was significantly greater ($p < 0.05$) than HFD control, significantly lower than NFD control, but not significantly different from the orlistat group (Table 9).

Table 8: Liver function enzymes of rats

	ALT (U/L)	AST (U/L)	ALP (U/L)
NFD control	21.89±0.29[a]	19.17±0.12[a]	48.38±0.11[a]
HFD control	30.60±1.40[b]	27.57±0.22[b]	53.60±0.02[b]
Orlistat (20mg/kg)	21.20±0.34[a]	14.82±0.23[c]	50.42±0.09[c]
***Z. officinale* (200mg/kg)**	23.68±0.26[c]	14.68±0.29[c]	48.33±0.12[a]
***Z. officinale* (400mg/kg)**	24.80±0.33[c]	19.30±0.24[a]	50.35±0.10[c]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

Table 9: Bilirubin, total protein and albumin concentrations of rats

	Bilirubin(µmol/L)	Total protein (g/dL)	Albumin (g/dL)
NFD control	18.24±0.73[a]	7.17±0.35[ac]	4.14±0.02[a]
HFD control	34.73±0.81[b]	6.01±0.06[b]	2.74±0.01[b]
Orlistat (20mg/kg)	21.83±0.42[c]	7.03±0.01[c]	3.94±0.02[c]
***Z. officinale* (200mg/kg)**	18.37±0.79[a]	7.42±0.08[a]	3.80±0.03[c]
***Z. officinale* (400mg/kg)**	15.41±0.41[d]	7.28±0.12[ac]	3.72±0.06[c]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.7 Cardiac function tests

The activity of creatine kinase in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control and the orlistat group but not significantly different from NFD control (Table 10).

The activity of lactate dehydrogenase in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control but significantly higher ($p < 0.05$) than NFD control. Lactate dehydrogenase of *Zingiber officinale* (200mg/kg) was significantly lower ($p < 0.05$) than *Zingiber officinale* (400mg/kg) but was not significantly different from the orlistat group (Table 10).

Table 10: Cardiac function enzymes of rats

	Creatinine kinase (U/L)	Lactate dehydrogenase (U/L)
NFD control	10.47±0.19[a]	23.25±1.42[a]
HFD control	16.56±0.18[b]	84.93±0.80[b]
Orlistat (20mg/kg)	12.62±0.03[c]	58.74±0.65[c]
***Z. officinale* (200mg/kg)**	10.60±0.27[a]	58.87±0.43[c]
***Z. officinale* (400mg/kg)**	11.26±0.18[a]	75.48±0.23[d]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.8 In vivo antioxidant parameters of rats

For both the liver and heart, GSH concentration in *Zingiber officinale* leaves extract groups was significantly higher ($p < 0.05$) than HFD control but significantly lower ($p < 0.05$) than NFD control. However, GSH concentration of *Zingiber officinale* (400mg/kg) was significantly higher ($p < 0.05$) than *Zingiber officinale* (200mg/kg) and the orlistat group (Tables 11 and 12).

For liver MDA concentration, *Zingiber officinale* leaves extract groups were significantly lower ($p < 0.05$) than HFD control, while *Zingiber officinale* (200mg/kg) had significantly lower ($p < 0.05$) MDA concentration than *Zingiber officinale* (400mg/kg), NFD control, and the orlistat group (Table 11).

In the heart, MDA concentration of *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control but significantly higher ($p < 0.05$) than NFD control and the orlistat group. Also, *Zingiber officinale* (400mg/kg) had significantly lower ($p < 0.05$) MDA concentration than *Zingiber officinale* (200mg/kg) (Table 12).

Liver GPx activities in *Zingiber officinale* leaves extract groups were significantly higher ($p < 0.05$) than HFD control but not significantly different from NFD control. GPx activity of *Zingiber officinale* (400mg/kg) was significantly higher ($p < 0.05$) than *Zingiber officinale* (200mg/kg) and the orlistat group (Table 11).

Heart GPx activities in *Zingiber officinale* leaves extract groups were significantly higher ($p < 0.05$) than HFD control, significantly lower ($p < 0.05$) than NFD control but not significantly different from the orlistat group (Table 12).

Table 11: Antioxidant parameters in liver of rats

	GSH (ng/mg protein)	MDA (nmoles /mg protein)	GPx (nmoles/ min/ mg protein)
NFD control	1.94 ± 0.01^{a}	0.89 ± 0.01^{a}	0.81 ± 0.02^{ad}
HFD control	0.82 ± 0.01^{b}	2.53 ± 0.13^{b}	0.24 ± 0.02^{b}
Orlistat (20mg/kg)	1.67 ± 0.01^{c}	0.64 ± 0.02^{c}	0.73 ± 0.02^{ac}
***Z. officinale* (200mg/kg)**	1.44 ± 0.01^{d}	0.45 ± 0.03^{d}	0.76 ± 0.01^{ac}
***Z. officinale* (400mg/kg)**	1.74 ± 0.02^{e}	0.90 ± 0.01^{a}	0.87 ± 0.01^{d}

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

Table 12: Antioxidant parameters in heart of rats

	GSH (ng/mg protein)	MDA (nmoles /mg protein)	GPx (nmoles/ min/ mg protein)
NFD control	1.49 ± 0.02^{a}	0.46 ± 0.03^{a}	1.73 ± 0.07^{a}
HFD control	0.65 ± 0.02^{b}	1.80 ± 0.01^{b}	0.56 ± 0.02^{b}
Orlistat (20mg/kg)	1.16 ± 0.01^{c}	0.42 ± 0.01^{a}	1.21 ± 0.04^{c}
***Z. officinale* (200mg/kg)**	1.05 ± 0.01^{d}	0.64 ± 0.01^{c}	1.04 ± 0.01^{c}
***Z. officinale* (400mg/kg)**	1.31 ± 0.02^{e}	0.55 ± 0.01^{d}	0.96 ± 0.02^{c}

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.9 Adipokines and cytokines concentrations

There was no significant difference ($p > 0.05$) in leptin concentration of *Zingiber officinale* leaves extract groups, NFD control, HFD control, and the orlistat group. The concentration of adiponectin in *Zingiber officinale* leaves extract groups was significantly higher ($p < 0.05$) than HFD control and the orlistat group but significantly lower than NFD control. Also, adiponectin concentration of *Zingiber officinale* (400mg/kg) was significantly higher ($p < 0.05$) than *Zingiber officinale* (200mg/kg) (Table 13).

The concentration of TNFα in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control, NFD control, and the orlistat group. Also, TNFα of *Zingiber officinale* (400mg/kg) was significantly higher ($p < 0.05$) than *Zingiber officinale* (200mg/kg) (Table 13).

The concentration of IL-6 in *Zingiber officinale* leaves extract groups was significantly lower ($p < 0.05$) than HFD control, not significantly different from the orlistat group, but significantly higher than NFD control.

Table 13: Adipokines and cytokines concentrations of rats

	Leptin (pg/mL)	Adiponectin (ng/mL)	TNFα (ng/mL)	IL-6 (ng/mL)
NFD control	213.83±4.12[a]	2.63±0.01[a]	179.94±1.37[a]	264.86±0.74[a]
HFD control	213.51±2.19[a]	0.63±0.01[b]	296.09±1.41[b]	395.43±1.08[b]
Orlistat (20mg/kg)	222.02±2.52[a]	1.93±0.04[c]	206.85±1.32[c]	301.35±0.38[c]
***Z. officinale* (200mg/kg)**	223.60±3.34[a]	2.28±0.08[d]	151.29±2.05[d]	302.67±0.89[c]
***Z. officinale* (400mg/kg)**	222.77±0.58[a]	2.49±0.01[e]	167.50±1.12[e]	300.18±2.31[c]

Results are shown as mean±SEM (n = 6). Different alphabets in same column signifies significant difference between means ($p < 0.05$)

3.10 Liver and kidney histology of rats

3.10.1 Liver histology

NFD control showed normal hepatic histology with clearly delineated portal triads, central veins, and spirally arranged hepatocytes. Hepatocytes featured large nuclei with conspicuous nucleoli. No obvious pathological changes or hepatocyte degeneration were observed in other groups (Plate 1).

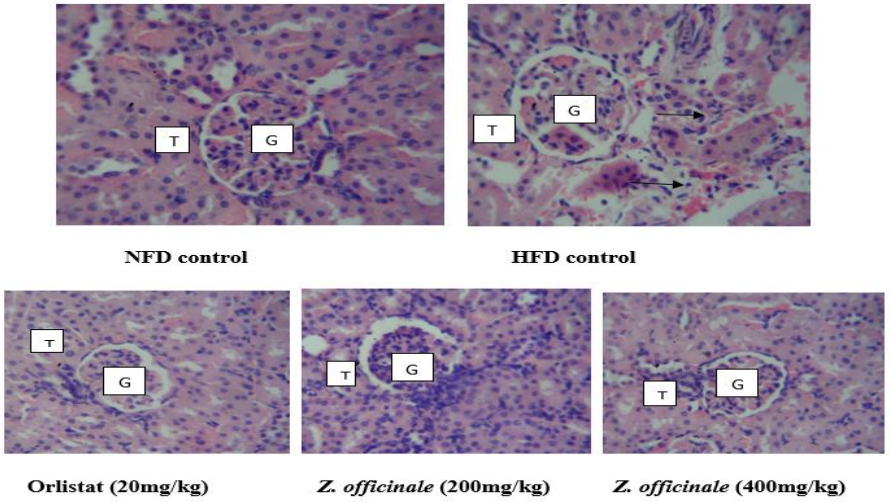

Plate 1: Histological changes in liver of experimental groups. H&E; Magnification = x400. Black arrow– hepatic nuclei; white arrow– portal triad

3.10.2 Kidney histology

NFD control showed normal renal histology with distinct renal corpuscles consisting of intact glomeruli and narrow Bowman spaces surrounding the glomeruli. Additionally, numerous intact tubules (proximal and distal tubules) were seen in the kidney cortex. Extract and orlistat treated groups also showed mostly intact renal histology. However, HFD control showed mild hemorrhage into the tissue parenchyma with mild inflammatory infiltrate (Plate 2).

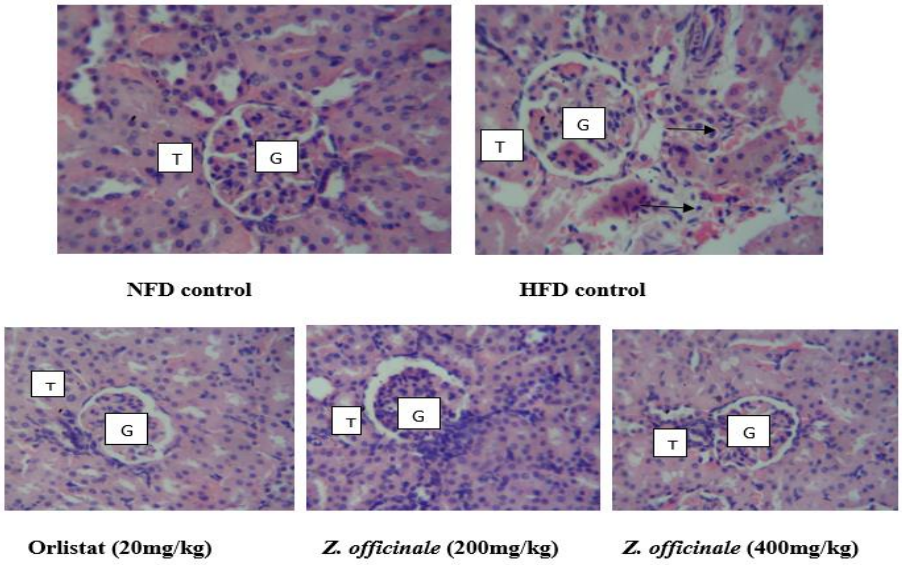

Plate 2: Histological changes in kidney of experimental groups. H&E; Magnification = x400. G – glomerulus; T – renal tubules; arrows – mild haemorrhage with inflammatory infiltrate

DISCUSSION

This study was conducted to evaluate the anti-obesity effect of methanol leaves extract of *Zingiber officinale* in Wistar rats fed with HFD. HFD-induced obesity in rats is regarded as a reliable technique for studying anti-obesity activity[34].

In this research, consumption of calorically dense HFD led to an increase in body weight, weights of epididymal and retroperitoneal fats. However, *Z. officinale* leaves extract-treated groups had significantly lower ($p < 0.05$) body weights than HFD control but were not significantly different from NFD control and the orlistat group. There was also a significant decline ($p < 0.05$) in adiposity of *Z. officinale* leaves extract groups compared to HFD control, which was comparable to that of the reference drug, Orlistat. Orlistat is a derivative of lipstatin that inhibits the activity of pancreatic lipase, resulting in reduced dietary fat absorption[35]. Similarly, Nazish *et al.* [36] reported a significant decrease in body weights and adiposity of rats fed with HFD and treated with *Z. officinale* rhizomes compared to HFD control. The ability of methanol leaves extracts of *Z. officinale* to decrease body weight and adiposity of rats might be attributed to singular or synergistic activities of the phytochemicals in it. Leaves of *Z. officinale* have been reported to be rich in flavonoids, tannins, saponins, and glycosides[24]. Some of these compounds have anti-obesity properties. Specifically, flavonoids have been found to possess anti-obesity effects by inhibiting adipogenesis and lipogenesis[37]. In addition, saponins have been found to exhibit anti-obesity effects by inhibiting adipocyte hypertrophy[38].

Lipid profile, CRI, and AI have been shown to be significant determinants of metabolic disturbances such as atherosclerosis, cardiovascular diseases, hypertension, and dyslipidemia. Any increase in concentrations of lipids raises the risk for atherosclerotic plaques, cardiovascular diseases, and endothelial dysfunction[39]. In this study, methanol leaves extract of *Z. officinale* demonstrated an anti-dyslipidemic effect by restoring the lipid profile, lowering AI and CRI in the rats. These results support those of Ramadan *et al.*[40] in which *Z. officinale* rhizomes produced anti-dyslipidaemic effect in HFD induced rats.

A reliable and fast method for the determination of obesity in rats was described by Lee [41] and was named "Lee index". Lee index and fat mass have a positive correlation. Rats with Lee's index equal to or greater than 0.3 are considered obese, according to Malafaia *et al.*[42] BMI is another anthropometric parameter that can be used to estimate obesity in rats[43]. Results obtained indicated that *Z. officinale* leaves extract-treated groups had significantly lower ($p < 0.05$) BMI and Lee's index than HFD control but were not significantly different from NFD control and the orlistat group. Furthermore, *Z. officinale* leaves extract, orlistat, and NFD groups had Lee's index below 0.3 and can be regarded as non-obese, whereas HFD control had Lee's index higher than 0.3 and may be regarded as obese rats based on the explanation by Malafaia *et al.*[42].

Results from this study showed that the extract of *Z. officinale* leaves exhibited anti-obesity activity in rats without affecting feed consumption. This is because there was no significant difference in daily feed consumption of *Z. officinale* (methanol leaves extract) groups compared to HFD control. Research by Nazish *et al.* [36] also showed that *Z. officinale* rhizomes also produced anti-obesity effect in rats fed with HFD without altering feed consumption of the rats.

HFD is known to affect liver metabolism, causing steatosis, which is a complicated disorder related to mitochondrial changes and increased formation of reactive oxygen species[44]. This concept explains the significant reduction ($p < 0.05$) in AST, ALT, ALP, bilirubin with an increase in the concentration of albumin and total proteins of the *Z. officinale* leaves extract, NFD control, and orlistat groups compared to HFD control. AST activity and albumin concentration of *Z. officinale* leaves extract groups were not significantly different from the orlistat group, and *Z. officinale* leaves extract groups had significantly higher ($p < 0.05$) ALT than NFD control. Similar findings were made by Vanissa *et al.* [45] where treatment of HFD fed rats with *Z. officinale* rhizomes extract restored liver function of rats to normal.

Obesity and dyslipidaemia are risk factors for cardiovascular diseases[46]. This research showed that there was a significant reduction ($p < 0.05$) in the activities of cardiac function enzymes (creatine kinase and lactate dehydrogenase) in *Z. officinale* leaves extract, NFD control, and orlistat groups in comparison with HFD control. Creatine kinase of *Z. officinale* leaves extract groups was significantly lower than the orlistat group but was not significantly different from NFD control. Cardioprotective effects of *Z. officinale* rhizomes extract in rats have been reported by Ojo *et al.*[47].

HFD induced obesity may cause renal injury and inflammation [48]. In this study, renal injury of rats induced by HFD was ameliorated by the methanol leaves extract of *Z. officinale* and orlistat in their respective groups. This explains the observed significant decrease ($p < 0.05$) in kidney function markers (urea and creatinine) in the leaves extract of *Z. officinale*, NFD control, and orlistat groups in comparison with HFD control. Urea levels for the methanol leaves extract of *Z. officinale* groups were not significantly different from orlistat and NFD control, whereas creatinine levels for the methanol leaves extract of *Z. officinale* groups were significantly higher than orlistat and NFD control. Low bicarbonate concentration is synonymous with metabolic acidosis, which indicates kidney failure[49]. The result from this study also revealed that bicarbonate concentrations in *Zingiber officinale* leaves extract groups were significantly higher ($p < 0.05$) than HFD control, orlistat, and NFD control. Furthermore, renal histology revealed that the methanol leaves extract of *Z. officinale* and orlistat groups had normal kidney histology similar to NFD control, whereas HFD control had inflammation and mild hemorrhage. These findings are in agreement with those of Vanissa *et al.*, in which the extract of *Z. officinale* rhizomes protected the kidney of rats fed with HFD.

Research has shown that the consumption of HFD causes oxidative stress because it attenuates the hepatic antioxidant enzyme system and increases the levels of products of lipid peroxidation in the liver and plasma[50]. Methanol leaves extract of *Z. officinale* protected against oxidative damage

caused by HFD in rats by significantly ($p < 0.05$) raising the activity of an antioxidant enzyme, GPx, and the concentration of GSH (a powerful antioxidant that directly quenches ROS), thus leading to lowered concentration of MDA (a product of lipid peroxidation) in the liver and heart of rats when compared to HFD control. Liver and heart GSH levels of *Z. officinale* leaves extract groups were significantly lower ($p < 0.05$) than NFD control. Heart MDA levels for *Z. officinale* leaves extract groups were significantly higher ($p < 0.05$) than orlistat and NFD control. No significant difference was observed in the liver GPx activities of *Z. officinale* leaves extract groups and NFD control. These results support that of Ramadan *et al.* [40] where *Z. officinale* rhizomes led to a reduction in oxidative stress of rats fed with HFD.

Methanol leaves extract of *Z. officinale* also showed a protective effect against inflammation induced by HFD. This is seen from the significant reduction ($p < 0.05$) in the concentration of proinflammatory cytokines (TNFα and IL-6) in comparison with HFD control. TNFα levels of *Z. officinale* leaves extract groups were significantly lower ($p < 0.05$) than orlistat and NFD control. However, IL-6 levels of *Z. officinale* leaves extract groups were significantly higher ($p < 0.05$) than NFD control but not significantly different from the orlistat group.

Leptin (an appetite-suppressing hormone) is synthesized by adipocytes, and it acts on the hypothalamus, resulting in reduced food intake[51]. There was no significant difference ($p > 0.05$) in leptin concentrations of all groups. This could justify why feed consumption of rats (appetite) in various groups did not differ significantly. This result supports that of Hussain *et al.* [52] whose work also showed no significant difference in leptin levels of HFD- obese control rats and other non-obese rats. Adiponectin is produced largely in adipocytes. One of its major physiological effects is that it decreases gluconeogenesis and lipogenesis in the liver, thereby resulting in decreased blood glucose and fat concentrations[53]. Unlike leptin, adiponectin levels are lowered in obesity. There is an inverse relationship between adiponectin levels and the percentage of body fat[54]. Thus, in this study, adiponectin levels of *Z. officinale* leaves extract groups were significantly higher ($p < 0.05$) than HFD control. Also, adiponectin levels of *Z. officinale* leaves extract groups were significantly lower ($p < 0.05$) than NFD control but significantly higher ($p < 0.05$) than the orlistat group.

CONCLUSION

This study has shown that the methanol leaves extract of *Z. officinale* possesses significant anti-obesity properties that are similar to orlistat (a reference anti-obesity drug). This anti-obesity effect might be mediated through the regulation of fat metabolism. Additionally, the methanol leaves extract of *Z. officinale* protected rats against oxidative damage, inflammation, liver, kidney, and cardiac toxicity induced by HFD. However, further study to characterize the active compounds in the extract is recommended.

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AUTHOR CONTRIBUTIONS

OI contributed to conceptualization, methodology, data interpretation and writing a draft of the manuscript. ESU contributed to investigation, methodology, supervision and revision of the manuscript. IOA contributed to methodology and revision of the manuscript. NEO participated in methodology, data analysis and interpretation. OLA contributed to methodology and data analysis. All the authors read and approved the final copy of the manuscript.

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COMPETING INTERESTS

No competing interests were declared by the authors

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أظهر مستخلص أوراق الميثانول من (ginger) *Zingiber officinale* تأثيرًا مضادًا للسمنة في فئران Wistar التي تتغذى على نظام غذائي عالي الدهون

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ملخص

قيمت هذه الدراسة خاصية مكافحة السمنة لمستخلص الميثانول من أوراق *Zingiber officinale* في فئران Wistar. تم توزيع ثلاثين من ذكور الجرذان في خمس مجموعات مع ستة فئران في كل مجموعة وعولجت مجموعات مختلفة بنظام غذائي طبيعي للدهون (NFD، نظام غذائي عالي الدهون 20) HFD + orlistat (HFD), مجم / كجم) p.o، HFD + 200) *Zingiber officinale* مجم / كجم) p.o و HFD + *Zingiber officinale*). بعد جميع الإدارات ، تم التضحية بالحيوانات عن طريق خلع عنق الرحم وأجريت تحليلات كيميائية حيوية مختلفة. أظهرت النتائج أنه كان هناك انخفاض كبير (p ug 0.05) في وزن الجسم والسمعة في مجموعات *Zingiber officinale* و NFD و orlistat مقارنة مع التحكم في HFD. ومع ذلك، لم يكن هناك فرق كبير في أوزان الجسم للفئران في مجموعات *Zingiber officinale* مقارنة مع مجموعات NFD للتحكم و orlistat. علاوة على ذلك، كان لدى الفئران في مجموعات *Zingiber officinale* تركيزات دهنية طبيعية، وحالة مضادة للأكسدة، والأدييوكينات، والسيتوكينات، والكبد، معلمات وظائف الكلى والقلب التي يمكن مقارنتها مع أورليستات والتحكم الطبيعي ولكن على النقيض من التحكم HFD. تشير نتائج الدراسة إلى أن أوراق *Zingiber officinale* لها خصائص كبيرة مضادة للسمنة ومضادات الأكسدة ومضادة للالتهابات.

الكلمات الدالة: نبات الزنجبيل، وزن الجسم، الأنسجة الدهنية، السيتوكينات، أورليستات، الأدييوكينات، مضادات الأكسدة.

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An Insight into the Structure-Activity Relationship of Antimicrobial Peptide Brevinin

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ABSTRACT

Numerous amphibian species, particularly those of the genus Rana, have been found to produce linear, amphiphilic, and cationic antimicrobial peptides (AMPs). Such AMPs are gaining more attention in pharmaceutical applications due to their principal method of action, which involves penetrating and rupturing the intended cell membranes with relatively low resistance. Brevinin is a large family of AMPs extensively studied during the last few decades, primarily consisting of two groups of peptides: Brevinin-1 and Brevinin-2. These peptides are cationic and establish secondary structures in the biological membrane environment. In this discussion, we explore the effects of structural parameters (net charge, hydrophobicity, amphiphilicity, helicity, peptide length, etc.) of Brevinin on their antimicrobial activity. As a general rule, an increased net charge tends to enhance antimicrobial activity. However, it is important to note that excessive net charges can also elevate hemolytic activity. The amino acid composition significantly influences hydrophobicity and helicity, which, in turn, impact the activity of the peptides. Moreover, these structural parameters are interconnected; modifying one parameter will affect others. Striking an optimal balance in these factors will provide a Brevinin analog with the highest antimicrobial activity and the lowest hemolytic activity.

Keywords: Antimicrobial peptides; Brevinin; Helicity; Hydrophobicity; Net charge; Hemolytic activity.

1. INTRODUCTION

The irrational use of antibiotics contributes to antimicrobial resistance in infectious pathogens, posing a severe global public health concern [1]. Increased antimicrobial resistance has resulted in the failure of traditional medicine to treat conditions effectively, heightened infection risks, prolonged hospital admissions, and, ultimately, an economic burden on nations [2, 3]. Therefore, it is vitally necessary to develop active antimicrobial compounds with lower resistance levels.

Antimicrobial peptides (AMPs) play a significant role in combating resistant microbes due to their rapid and broad-spectrum effectiveness against fungi, viruses, and both Gram-positive and Gram-negative bacteria [4, 5]. In contrast to conventional antibiotics, AMPs bind to the bacterial membrane, causing disruption rather than targeting a specific site. The barrel-stave, carpet, or toroidal models are employed to describe the membrane disruption caused by AMPs [6-8], making it challenging for microorganisms to develop resistance against them [9].

AMPs are typically small, naturally occurring peptide molecules consisting of 10 to 50 amino acids. Most AMPs carry a net positive charge at physiological pH due to the presence of basic amino acids, facilitating electrostatic interactions with the membranes of negatively charged microorganisms [10]. An important structural feature of these peptides is amphiphilicity, characterized by the presence of hydrophobic residues on one side and hydrophilic residues on the other side of the molecule [11]. Amphiphilicity also aids in binding to the hydrophobic and hydrophilic regions of the target pathogen. In the presence

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of lipid membranes, many AMPs adopt a distinct secondary structure, such as α-helix or β-sheet, essential for antibacterial activity [12, 13].

AMPs are ubiquitous in almost every living organism, serving as a component of their innate immune system. Extracted from the skin of amphibians, these AMPs have demonstrated superior efficacy against microorganisms, offering a potential solution to current antimicrobial resistance problems [14].

Amphibian AMPs are categorized into peptide families, including brevinins, cathelicidin, temporins, esculentin, ranatuerin, etc., based on shared structural properties and their ability to combat pathogens [15]. Brevinin (Figure 1) is a crucial amphibian AMP family isolated from the Ranidae, exhibiting high biological activities and distinctive structural properties [16]. Originally identified in Rana brevipoda porsa [17], hundreds of Brevinin peptides have been discovered and their data deposited in the database [18]. These peptides exhibit various bioactivities, encompassing antimicrobial, anticancer, hypoglycemic, anti-inflammatory, and more [19].

Researchers have designed numerous Brevinin analogs, aligning with common AMP features and the predicted secondary structure of peptides, to explore the structure-activity relationship and enhance their antimicrobial activity. For instance, Lin et al. extracted Brevinin-2GUb from the skin secretion of Hylarana guentheri, producing analogs to investigate its cationic activity [20]. The augmentation of cationic charges significantly enhances the compound's bioactivity while mitigating its toxicity effects. AMPs exhibit various structural parameters (amphiphilicity, net charge, charge density, length, hydrophobicity, hydrophobic moment, and helicity) influencing antimicrobial activity. Hence, this study delves into the structural features of Brevinin analogs and the impact of structural changes on their antimicrobial activity. Understanding the structural activity relationship of Brevinins is crucial for identifying the most effective peptide to serve as a template for developing new antimicrobial agents with medicinal properties.

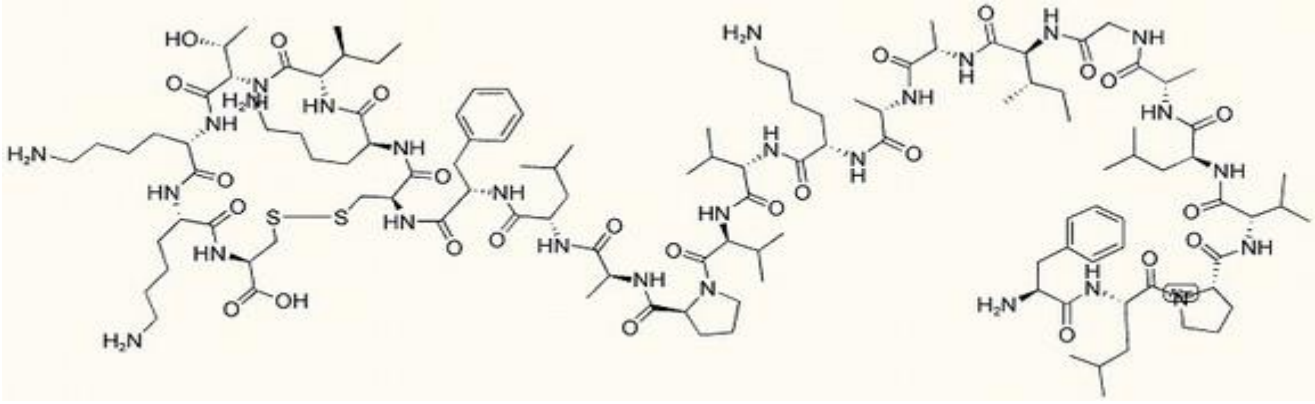


Figure 1. Chemical structure of Brevinin-1

2. Importance of SAR study of Brevinin

A structure-activity relationship (SAR) study is crucial for understanding the correlation between structural parameters and the antimicrobial activity of peptides. Researchers can create synthetic analogs of parent peptides to enhance antimicrobial efficacy. For example, synthetic hybrid peptides derived from natural indolicidin and ranalexin exhibit greater antibacterial activity compared to their parent analogs against Streptococcus pneumoniae [21]. Larger antimicrobial peptides may demonstrate increased hemolytic tendencies, whereas shorter peptides with higher cationic content tend to reduce hemolytic activity [22].

An in-depth exploration of peptide structure is essential

for the rational design of α-helical AMPs with heightened antimicrobial activity and specificity [23]. Examining the SAR of AMPs is also vital for comprehending the diverse mechanisms responsible for the antimicrobial activities of peptides [24]. It is imperative for researchers to expand SAR studies to unearth novel peptide analogs with enhanced antimicrobial activity. Brevinin, a substantial group of AMPs, exhibits a broad-spectrum activity against bacteria, viruses, fungi, and other parasites (Table 1). Understanding the structure-activity relationship of Brevinin is pivotal for discovering more bioactive analogs, contributing to advancements in medical science.

Table 1. Some notable Brevinin family AMPs with their sequences, sources, and major bioactivities

Name	Length	Amino Acid Sequence*	Bioactivities	Source	References
Brevinin-1	24	FLPVLAGIAAKVVPALFCKITKKC	Antibacterial, high hemolytic activity	*Rana brevipoda porsa*	[17]
Brevinin-1AVa	17	FLPLLAASFACTVTKKC	Antibacterial	*Rana arvalis*	[25]
Brevinin-1Ba	24	FLPFIAGMAAKFLPKIFCAISKKC	Antibacterial	*Lithobates berlandieri*	[26]
Brevinin-1BYa	24	FLPILASLAAKFGPKLFCLVTKKC	Antibacterial, antifungal and hemolytic activity	*Rana boylii*	[27]
Brevinin-1CDYa	20	LLSLALAALPKLFCLIFKKC	Antibacterial and weak hemolytic activity	*Rana chensinensis*	[28]
Brevinin-1CG1	24	FLSTALKVAANVVPTLFCKITKKC	Antibacterial, antifungal and low hemolytic activity	*Amolops chunganensis*	[29]
Brevinin-1CSa	24	FLPILAGLAAKIVPKLFCLATKKC	Antibacterial and strong hemolytic activity	*Rana cascadae*	[30]
Brevinin-1DYb	20	FLSLALAALPKLFCLIFKKC	Antibacterial, antifungal, anticancer, candidacidal and strong hemolytic activity	*Rana dybowskii*	[31]
Brevinin-1E	24	FLPLLAGLAANFLPKIFCKITRKC	Antibacterial	*Pelophylax saharicus*	[32]
Brevinin-1HN1	24	FLPLIASLAANFVPKIFCKITKKC	Antibacterial, antifungal, candidacidal and low hemolytic activity	*Odorrana hainanensis*	[33]
Brevinin-1HSa	24	FLPAVLRVAAKIVPTVFCAISKKC	Antibacterial activity	*Odorrana hosii*	[34]
Brevinin-1ITa	20	IVPFLLGMVPKLVCLITKKC	Antibacterial, cytotoxic and hemolytic activity	*Rana italica*	[35]
Brevinin-1OKa	22	FFGSMIGALAKGLPSLISLIKK	Antibacterial	*Rana okinavana*	[36]
Brevinin-1OKc	22	FFGSIIGALAKGLPSLISLIKK	Antibacterial	*Rana okinavana*	[36]
Brevinin-1Pa	24	FLPIIAGVAAKVFPKIFCAISKKC	Antibacterial, antifungal and candidacidal activity	*Rana pipiens*	[26]
Brevinin-1PLb	24	FLPLIAGLAANFLPKIFCAITKKC	Antibacterial, antifungal and candidacidal activity	*Lithobates palustris*	[37]
Brevinin-1Ra	24	VIPFVASVAAEMMQHVYCAASRRC	Antibacterial	*Pelophylax ridibundus*	[38]

Name	Length	Amino Acid Sequence*	Bioactivities	Source	References
Brevinin-1Sa	24	FLPAIVGAAGQFLPKIFCAISKKC	Antibacterial and antifungal activity	*Rana sphenocephala*	[39]
Brevinin-1SE	23	FLPLVRGAAKLIPSVVCAISKRC	Antibacterial activity	*Rana sevosa*	[40]
Brevinin-1SN1	24	FLPAVLKVAAHILPTAICAISRRC	Antibacterial and hemolytic activity	*Hylarana spinulosa*	[41]
Brevinin-1SPa	24	FFPIIAGMAAKLIPSLFCKITKKC	Antibacterial, antifungal, candidacidal and hemolytic activity	*Lithobates septentrionalis*	[42]
Brevinin-1T	20	VNPIILGVLPKFVCLITKKC	Antibacterial, high hemolytic activity	*Rana temporaria*	[43]
Brevinin-2	33	GLLDSLKGFAATAGKGVLQSLLSTASCKLA KTC	Antibacterial, high hemolytic activity	*Rana brevipoda porsa*	[17]
Brevinin-2DYe	37	GLFSVVTGVLKAVGKNVAKNVGGSLLEQL KCKISGGC	Antibacterial	*Rana dybowskii*	[31]
Brevinin-2GHb	30	GVITDALKGAAKTVAAELLRKAHCKLTNSC	Antibacterial	*Rana guentheri*	[44]
Brevinin-2GHc	31	SIWEGIKNAGKGFLVSILDKVRCKVAGGCN P	Antibacterial	*Hylarana guentheri*	[44]
Brevinin-2GRa	33	GLLDTFKNLALNAAKSAGVSVLNSLSCKLS KTC	Antibacterial, antifungal, candidacidal and hemolytic activity	*Odorrana grahami*	[45]
Brevinin-2ISa	33	SLLDTFKNLAVNAAKSAGVSVLNALSCKISR TC	Antibacterial, Antifungal and candidacidal activity	*Odorrana ishikawae*	[46]
Brevinin-2JD	33	GLLDTFKNLALNAAKSAGVSVLNSLSCKLS KTC	Antibacterial, antifungal, candidacidal and weak hemolytic activity	*Odorrana jingdongensis*	[47]
Brevinin-2LT	33	GLMSVLKKAGKHVAKNVAASLMDSLKCKI TGGC	Antibacterial	*Rana latastei*	[48]
Brevinin-2R	25	KLKNFAKGVAQSLLNKASCKLSGQC	Antibacterial, antifungal, anticancer, candidacidal activity	*Pelophylax ridibundus*	[49]
Brevinin-2Ra	29	GILDSLKNFAKDAAGILLKKASCKLSGQC	Antibacterial	*Pelophylax ridibundus*	[50]
Brevinin-2Ta	33	GILDTLKNLAKTAGKGILKSLVNTASCKLSG QC	Antibacterial, antifungal, candidacidal, anti-inflammatory and wound-healing activity	*Pelophylax kl. esculentus*	[51]
Brevinin-Eu	24	VIPFVASVAAEMMQHIFCAASRKC	Antibacterial, very low hemolytic activity	*Euphlyctis cyanophlyctis*	[52]
Brevinins-ALa	24	FLPMLAGLAANFLPKLFCKITKKC	Antibacterial, antifungal, and strong hemolytic activity	*Amolops loloensis*	[53]

*Refer to **table S1** in the appendix

3. Structural features of Brevinin

The Brevinin family is divided into two subfamilies: Brevinin-1 and Brevinin-2. Brevinin-1 (FLPVLAGIAAKVVPALFCKITKKC) comprises approximately 24 residues, while Brevinin-2 (GLLDSLKGFAATAGKGVLQSLLSTASCKLAKTC) has a length of approximately 33 residues. Brevinin-1 exists in two forms: cyclic and acyclic Brevinin-1 [36]. Acyclic

Brevinin-1 lacks Cys residues but has an amidated C-terminus, while cyclic Brevinin-1 features a disulfide bond at its C-terminus. Although the primary structure of Brevinins may vary among species, they typically contain a highly conserved portion in the 'Rana box' sequence. The Rana box is usually situated at the C-terminus, and its structure is [Cys18-(Xaa)4-Lys-Cys24] for Brevinin-1 [54]. While the length and amino acid composition of the peptides may vary, they all carry a net positive charge at neutral pH. Bacterial cell membranes, lacking neutral and zwitterionic lipids compared to mammalian cell membranes, make it easier for Brevinins to selectively attach to the anionic phospholipids of bacterial cell membranes [55].

Brevinins typically exist as randomly arranged coils in an aqueous solution but adopt an amphipathic α-helical shape in environments that mimic hydrophobic membranes, such as 50% trifluoroethanol (TFA) [56]. The peptide structure and the side chains of Brevinin-1 in a 33% trifluoroethanol solution are depicted in Figure 2. The α-helical structure is believed to disturb the phospholipid bilayer of the targeted membranes under such circumstances.

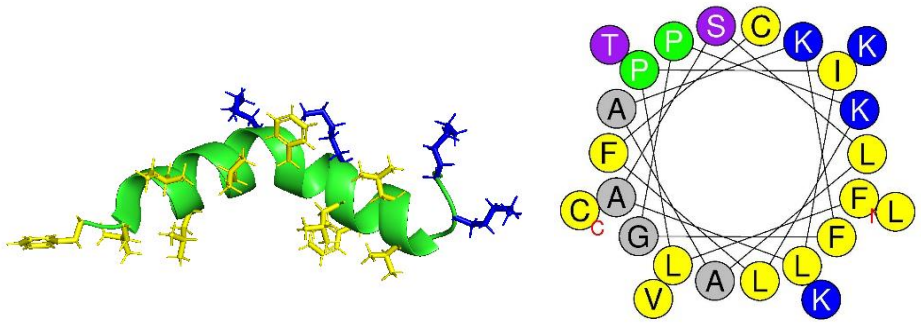


Figure 2. Structure of Brevinin-1BYa. (A) The solution NMR structure of Brevinin-1BYa in 33% trifluoroethanol (PDB ID: 6G4I) visualized in PyMOL (version 2.4.1.), (B) Helical wheel representation of Brevinin-1BYa obtained from HeliQuest server (https://www.heliquest.ipmc.cnrs.fr/). The positively charged, negatively charged, hydrophobic, and hydrophilic amino acids are each represented by a different color: blue, red, yellow, and purple, respectively. The letters 'N' and 'C' stand for the N-terminal and C-terminal, respectively.

3.1. Net charge

The peptides belonging to the Brevinin family are cationic. Typically, the positively charged residues (arginine, lysine), combined with negatively charged residues (aspartate, glutamate) in the peptide sequence, are totaled to determine the net charge of a peptide. The positive charge of the peptide molecule plays a crucial role in binding to the negatively charged surface of microorganisms [57]. Previous studies suggest that cationic peptides need a net charge of at least +2 to exhibit antibacterial effects effectively [58]. In general, higher net charge enhances the antibacterial action of peptides. For instance, Brevinin-2PTb (charge +5) demonstrates greater antimicrobial activity than Brevinin-2PTc (charge +4) and Brevinin-2HSa (charge +3) against E. coli 25726 [36]. However, it's essential to note that the impact of net charge is not always linear with activity. Another critical consideration is that an increased net charge of the peptide

also escalates hemolysis of human red blood corpuscles. Figure 3 illustrates the effects of net charge on the activity of various peptides [59, 60, 41].

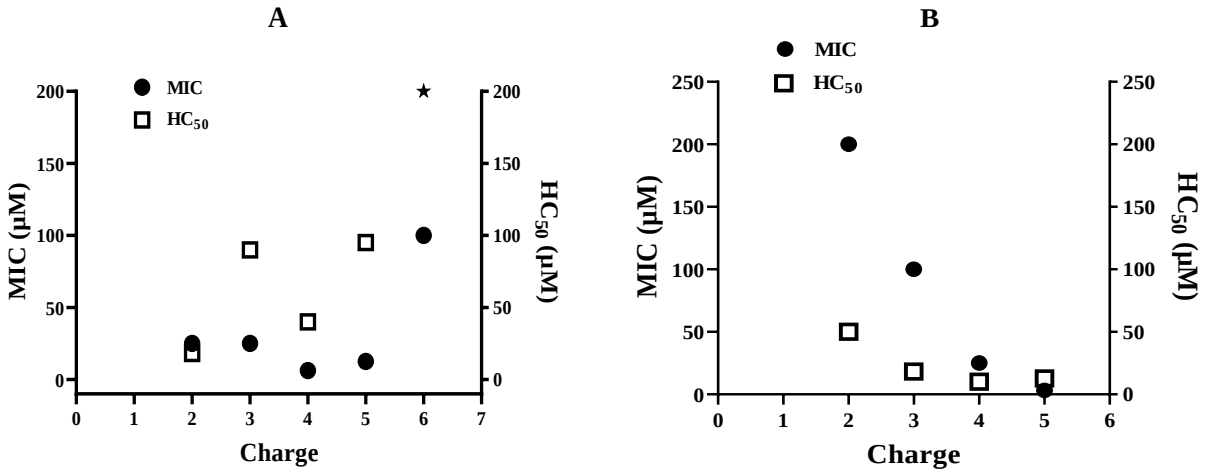


Figure 3. Relationship between charge and antimicrobial & hemolytic activity of Brevinin-2 related peptides (A) and Brevinin-1 peptides (B). The antimicrobial activity was measured in minimal inhibitory concentration (MIC) against *Staphylococcus aureus* 25923. The hemolysis of 50% human red blood cell concentration was considered as HC_{50}. ⋆ A marked point indicates a value greater than that point (>200µM).

Figure 3A illustrates that a peptide with a net charge of +4 exhibits the most potent antimicrobial activity, while one with a net charge of +2 shows hemolytic activity. Additional research by Islam et al. emphasizes that the optimal charge selection is crucial in peptide design to achieve maximum antimicrobial activity with minimal hemolysis [61]. In this context, we also observe that peptides with net charges of +3 and +5 display superior antimicrobial action with considerably low hemolytic activity.

Conversely, Figure 3B demonstrates that a peptide with a net charge of +5 has the best antimicrobial activity with the lowest hemolytic activity.

3.2. Hydrophobicity

The hydrophobicity of a peptide is measured by the solubility of its amino acids in water. The composition of a peptide determines its hydrophobicity, with a higher proportion of hydrophobic residues making the peptide more hydrophobic and vice versa.

The hydrophobicity of an AMP is a critical factor for its insertion into the pathogen's membrane. Trp, a hydrophobic amino acid, enhances its ability to bind to lipids, influencing the interfacial region of lipid bilayers [62]. A hydrophobicity threshold is presumed to play a role in selective bacterial membrane insertion and subsequent cell death. However, increased peptide hydrophobicity is associated with higher hemolytic activity [63]. In a previous investigation, Brevinin-2PRc and Brevinin-2PRd showed lower toxicity against red blood cells because their hydrophobicity decreased when Phe was replaced with Leu [64]. Another study demonstrated a linear relationship between hydrophobicity and hemolytic activity [65]. Thus, scrutinizing peptide hydrophobicity is crucial to avoid hemolysis. In Figure 3, we depict the relationship between the hydrophobicity of several Brevinin peptides and their

antimicrobial and hemolytic activity [66, 29]. The peptides share similar net charges and lengths, and their hydrophobicity values were calculated using HeliQuest.

In Figure 4A, it is shown that peptides with lower hydrophobicity values exhibit lesser antimicrobial activity, with the optimal action observed at 0.75; beyond this point, increasing hydrophobicity leads to a reduction in activity. The diminished activity of highly hydrophobic peptides is attributed to their self-aggregation, preventing them from passing through the microbial cell wall [63].

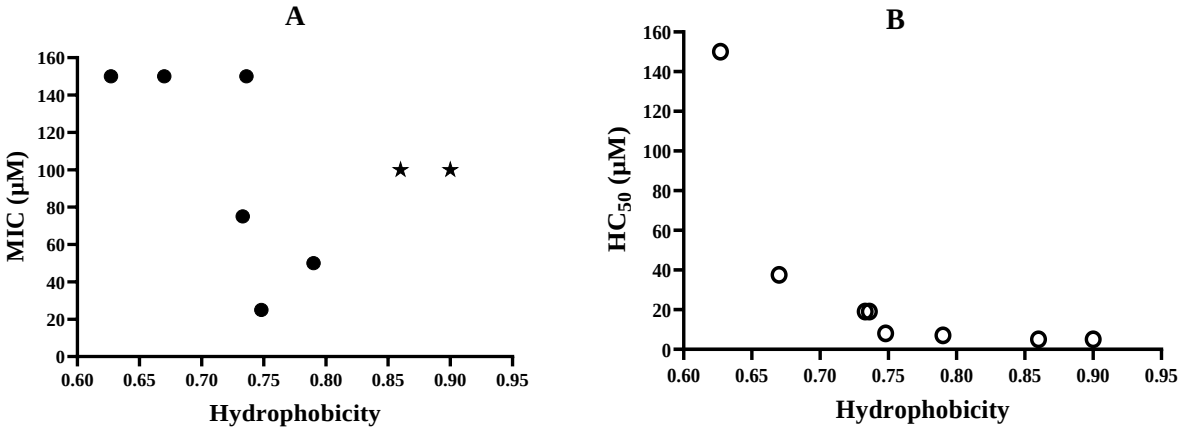


Figure 4. Relationship among hydrophobicity and antimicrobial activity (A) and hemolysis (B) of Brevinin peptides. The antimicrobial activity was measured in minimal inhibitory concentration (MIC) against *Escherichia coli* 25922. The *hemolysis of 50% human red blood cell concentration was considered as HC_{50}. Marked points indicate values greater than that point (>100µM).

Hemolytic activity demonstrates a direct proportionality to the hydrophobicity value of Brevinin peptides (Figure 4B). Given these two phenomena, it is advisable to design peptides with hydrophobicity that promotes antimicrobial activity while concurrently minimizing hemolytic activity.

3.3. Peptide length

The length and amino acid composition of Brevinins vary, but they all feature a distinctive "Rana Box" at the C-terminus, where positively charged residues cluster. Kumari and Nagaraj demonstrated that the disulfide bridge and the cationic cluster at the C-terminus of Brevinin 1E have no impact on antimicrobial activity but do affect hemolytic activity. Peptides lacking a disulfide bridge at the 'Rana Box' exhibit lower hemolytic activity due to a loss of rigidity [67]. Conversely, the activity of Brevinin-1GHa was diminished by removing the Rana Box from the C-terminus [68]. This reduction in activity may be attributed to a decrease in helicity. The N-terminal end of Brevinin-2GUb contains active fragments from the first to the nineteenth amino acids. However, the amino acid composition should offer sufficient hydrophobicity and net charge for interaction with the microbial membrane [20]. Despite being a 12-amino-acid-containing peptide, Brevinin-1OSf exhibits superior antimicrobial and lower hemolytic activity compared to Brevinin-1OS (24 amino acids) [69]. A C-terminus truncated peptide, Brevinin-2GK (1-25), demonstrated higher antibacterial activity than the parent Brevinin-2GK (33 residues), despite having less hemolytic activity. This suggests that the N-terminal helical segment of Brevinin-2GK is the primary cause of membrane rupturing [70].

It is evident that maintaining a balance between α-

helicity, positive charge, and hydrophobicity, rather than peptide length, is crucial for effective antibacterial activity.

3.4. Amphiphilicity

The amphiphilic nature of a peptide is essential for membrane activity [11]. This characteristic enables the interaction with lipid heads on the membrane and deep insertion into the membrane by segregating hydrophobic residues to one side of the helix.

Analog studies of Brevinin-1OS demonstrated a direct proportionality between amphiphilicity and the antimicrobial and hemolytic activity of peptides [69]. Figure 5 illustrates the linear relationship between the amphiphilicity index and the activity of four Brevinin-1 analogs (OSc, OSd, OSe, and OSf).

Among these peptides, the one with the lowest amphiphilicity exhibits the lowest activity (high MIC and HC50 values). Activity against microbes and red blood cells increases with the rise in amphiphilicity. Therefore, selecting a peptide with high antimicrobial activity and considerably low hemolytic activity is of utmost importance.

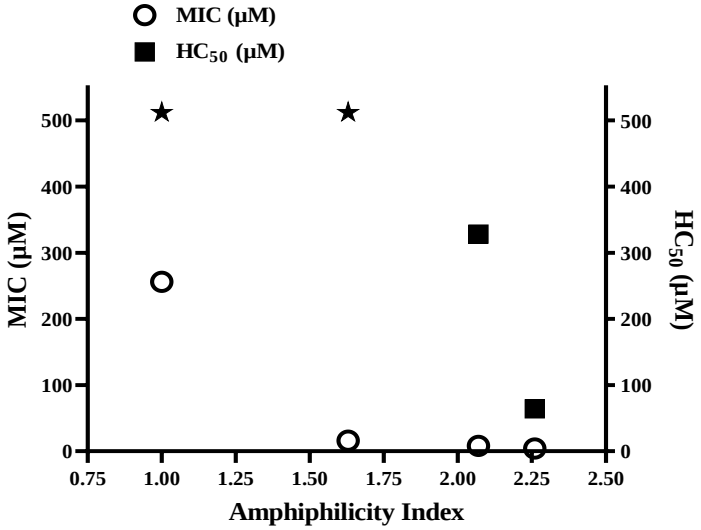


Figure 5. Relationship between amphiphilicity index and activity of four Brevinin-1 analogs. The amphiphilicity index is obtained from the Database of Antimicrobial Activity and Structure of Peptides (https://dbaasp.org/home). The MIC value was measured against *Staphylococcus aureus* (NCTC 10788), and HC_{50} was considered as the concentration required for the hemolysis of 50% of horse red blood cells. Marked points indicate values greater than that point (>512µM for HC_{50}).

3.5. Helicity

Helicity refers to the ability of an antimicrobial peptide to adopt a helical structure in a specific environment, such as negatively charged phospholipids in the bacterial cell membrane. Increased helicity enhances antimicrobial activity, selectivity, and stabilizes the α-helical structure. The combination of α-helicity and hydrophobicity is heightened by a cationic charge, which, in turn, increases hemolytic activity compared to antimicrobial activity [71].

While the structural properties of peptide α-helices may be crucial for bacterial cell death, their content may not directly correlate with antibacterial efficacy [72]. For

instance, OSd, due to the formation of a greater amount of α-helical composition than OSc, exhibits significantly increased antibacterial activity. Despite a more pronounced α-helical structure, OSd is less effective in inhibiting bacterial growth compared to both OSe and OSf, which have lower helicity [69].

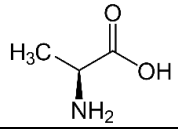
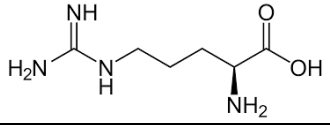
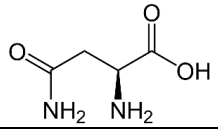
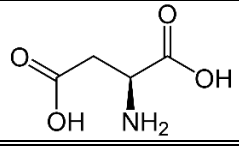
Analog studies of Brevinin-2GUb revealed reduced ability to generate a secondary structure (α-helix) in the analogs, resulting in lower activity on both the microbial surface and red blood cells [68]. A similar outcome was observed for B1CTcu1, where the peptide's binding affinity to the bacterial membrane and hemolytic activity were relatively low due to its diminished capacity to adopt an α-helical structure [73].

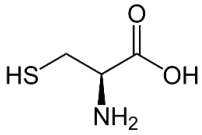
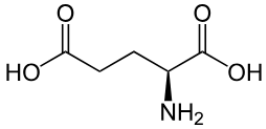
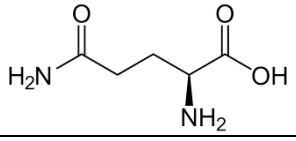
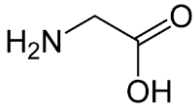
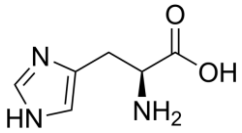
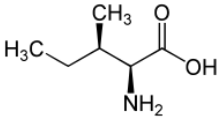
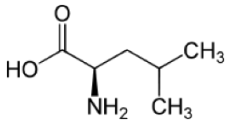
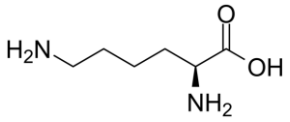
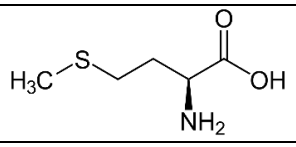
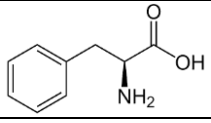
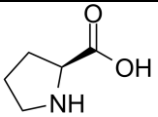
4. CONCLUSION

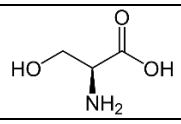
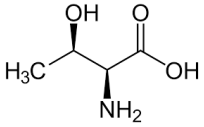
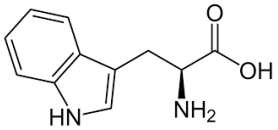
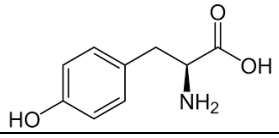
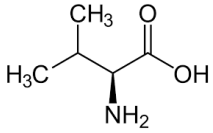
Antimicrobial peptides (AMPs) represent a promising alternative to traditional antibiotic molecules due to their remarkable activity against pathogenic species. Amphibian skin serves as an excellent source of animal AMPs, with Brevinin being a well-studied peptide over the past few decades. The activity of Brevinins can be modulated by manipulating various structural features. The net charge exhibits a direct relationship with activity, while hydrophobicity and α-helicity are predominantly associated with hemolytic activity. However, designing an active peptide requires consideration of multiple parameters. Positive charge, hydrophobicity, α-helicity, and amphipathicity interact intricately to delineate the cytolytic actions of Brevinins against both bacteria and mammalian cells. This study contributes to the design of novel, functional antimicrobial molecules with potential therapeutic applications.

Conflict of interest statement: The authors declared no conflict of interest.

Table S1: Amino acid coding system.

Amino acid name	Abbreviation	Single letter abbreviation	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic acid	Asp	D	

Amino acid name	Abbreviation	Single letter abbreviation	Structure
Cysteine	Cys	C	
Glutamic acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	

Amino acid name	Abbreviation	Single letter abbreviation	Structure
Serine	Ser	S	
Threonine	The	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

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نظرة ثاقبة على العلاقة بين التركيب والنشاط لببتيد بريفينين المضاد للميكروبات

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ملخص

تم العثور على العديد من أنواع البرمائيات، وخاصة تلك من جنس رنا، لإنتاج الببتيدات المضادة للميكروبات الخطية، البرمائية، والكاتيونية .(AMPs)تكتسب AMPsمزيدًا من الاهتمام في الاستخدامات الصيدلانية نظرًا لطريقة عملها الرئيسية، والتي تستلزم اختراق وتمزيق أغشية الخلايا المقصودة بمقاومة منخفضة نسبيًا Brevinin .هي عائلة كبيرة من AMPsتمت دراستها على نطاق واسع خلال العقود القليلة الماضية. تحتوي العائلة بشكل أساسي على مجموعتين من الببتيدات، وهما Brevinin-1 و .Brevinin-2هذه الببتيدات كاتيونية وتؤسس هياكل ثانوية في بيئة الغشاء البيولوجي. نناقش آثار المعلمات الهيكلية)شحنة صافية، كارهة للماء، amphiphilicity، حلزونية، طول الببتيد، إلخ (لبريفينين على نشاطها المضاد للميكروبات. كقاعدة عامة، ستؤدي زيادة الشحنة الصافية إلى زيادة نشاط مضادات الميكروبات. ومع ذلك، فإن الشحنات الصافية المفرطة تزيد أيضًا من النشاط الانحلالي. تؤثر تركيبة الأحماض الأمينية بشكل كبير على الكراهية للماء والطائرة، والتي بدورها تؤثر على نشاط الببتيدات. المعلمات الهيكلية مترابطة أيضًا ؛ تغيير معلمة واحدة سيؤثر على الآخرين. سيعطي التغيير الأمثل في هذه العوامل نظير Brevinin أعلى نشاط مضاد للميكروبات ولكن أقل نشاط انحلالي.

الكلمات الدالة: الببتيدات المضادة للميكروبات ؛ بريفينين، لولبية، كره الماء، صافي الشحن النشاط الانحلالي.

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Native Medicinal Plants (*Moringa oleifera* Lam, *Brucea javanica* (L.) Merr., *Eclipta prostrata* (L.), *Callisia fragrans* (Lindl.) Woodson, and *Zingiber zerumbet* (L.) Smith) in An Giang, Vietnam: A Preliminary Investigation for Rhabdomyosarcoma Treatments using in-vitro RD cell cytotoxicity test

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ABSTRACT

Cancer, one of the deadliest diseases worldwide, is projected to affect 30.2 million people by 2040. Among the various cancer types, rhabdomyosarcoma (RMS) is a unique tumor primarily impacting the muscular system of children. The current treatment for RMS has limited efficacy and numerous side effects, emphasizing the need for novel therapeutic approaches. This study investigates the potential treatment of the RMS cell line RD using extracts from five folklore-based medicinal plants in An Giang, Vietnam. The plants—Moringa oleifera Lam, Brucea javanica (L.) Merr., Eclipta prostrata (L.), Callisia fragrans (Lindl.) Woodson, and Zingiber zerumbet (L.) Smith—were extracted and fractionated using three solvents: ether, ethanol, and water. These fractions underwent phytochemical screening and cytotoxicity testing on the in-vitro RMS cell line RD. The results indicate that the ether fraction of Eclipta prostrata (L.) and the ether and ethanol fractions of Zingiber zerumbet (L.) Smith exhibit moderate cytotoxic effects on RD cell lines, with IC50 values of 37.08 ± 1.23 µg/mL, 23.15 ± 1.17 µg/mL, and 45.63 ± 2.39 µg/mL, respectively. These findings provide preliminary data for further in-depth research into the anticancer properties of these plants, which are widely grown in the South of Vietnam.

Keywords: rhabdomyosarcoma; *Moringa oleifera* Lam; *Brucea javanica* (L.) Merr.; *Eclipta prostrata* (L.); *Callisia fragrans* (Lindl.) Woodson; *Zingiber zerumbet* (L.) Smith; cytotoxicity; fractionation.

1. INTRODUCTION

According to the 2020 statistics from the Global Cancer Observatory (GCO), more than 19 million people worldwide have cancer, with Asia accounting for 49.3%, or about 9.5 million people[1]. Predictably, by 2040, the number of people with cancer is expected to reach 30.2 million. Currently, cancer ranks as the second leading cause of death worldwide, significantly impacting both the mental and physical lives of patients[2,3]. Among the more than 100 types of cancers, rhabdomyosarcoma (RMS) stands out as a special and rare tumor, primarily affecting the muscular system of children, especially the skeletal (voluntary) muscles[4]. Most cases of RMS are diagnosed in children aged ≤6, with risk factors and etiology remaining unknown. RMS is often sporadic, associated with familial syndromes, and can be categorized into different types:

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embryonal RMS (~60%), alveolar (~20%), pleomorphic (~10%), and spindle/sclerosing (~10%)[4]. This disease has been reported as the 3rd most common cancer and the most common soft tissue sarcoma in children[5]. Moreover, it can metastasize and develop into common cancers such as uterine cancer, stomach cancer, colon cancer, lymphoma, and limb cancer[6]. The current treatments for RMS, including surgery, radiation therapy, and chemotherapy (vincristine, actinomycin D, and cyclophosphamide/ifosfamide), yield poor and inadequate outcomes, especially in patients with metastatic and/or recurrent RMS[7,8]. For instance, the long-term event-free survival in metastatic RMS patients is <20%[9,10]. Last but not least, these treatments often result in numerous side effects such as fatigue, hair loss, nausea/vomiting, and diarrhea[11]. Therefore, it is crucial and urgent to search for novel treatments that offer better oncological outcomes with long-term safety for RMS patients.

To this end, a potential source for finding novel RMS chemotherapeutic treatments is the medicinal/herbal plants that grow wildly or are cultivated across ASEAN countries. Specifically, in Vietnam, a country with a rich source of medicinal plants, with over 7,000 described species, of which 3,830 species possess therapeutic properties[12]. In fact, in most Vietnamese hospitals, medicinal plants have been comprehensively utilized in complement with modern medicine, with over 700 official medical products containing herbal ingredients[13,14]. In the Mekong Delta, a green area in the South of Vietnam, nearly 1,000 medicinal plant species have been exploited, with 500-700 species originating from the forests in provinces with mountainous terrain, such as An Giang. An Giang, a frontier province bordered with Cambodia, is famous for its extremely diverse and rich vegetation, harboring numerous precious medicinal herbs[15,16]. Among them, five particular plants have gained much interest, namely moringa (Moringa oleifera Lam, Moringaceae, Chum Ngay [Vietnamese], MO), Macassar kernels (Brucea javanica (L.) Merr., Simaroubaceae, Xoan rung [Vietnamese], BJ), ink plant (Eclipta prostrata (L.), Asteraceae, Co muc [Vietnamese], EP), basket plant (Callisia fragrans (Lindl.) Woodson, Commelinaceae, Luoc vang [Vietnamese], CF), and shampoo ginger (Zingiber zerumbet (L.) Smith, Zingiberaceae, Gung gio [Vietnamese], ZZ), due to their well-known ethnopharmacology (i.e., folk remedies) in supporting cancer treatments[17–21]. In the literature, these medicinal plants have been widely reported for their diverse pharmacological effects, including anti-inflammation, anti-malarial, antibacterial, anti-diabetic, and anti-oxidant[18,21–24]. Nevertheless, limited information on the chemotherapeutic properties of these plants has been published, especially for RMS treatment.

Therefore, this study, for the first time, investigated the ability of these five plants to treat RMS in in-vitro cell culture settings. Prior to the cytotoxicity tests, the plants were extracted, and their phytochemical compositions were determined accordingly. We hypothesized that the plants would possess potential action on RMS, significantly contributing to the literature on novel ethnopharmacological medicinal plants in An Giang, Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

The samples of MO leaves, BJ seeds, EP leaves, ZZ roots, and CF leaves were collected in Tinh Bien district, An Giang province, Vietnam, in February 2021. The plants were identified by a botanical specialist with specialized botanical documents provided by the An Giang Forest Protection Department. Voucher specimens (CTUMP-111, CTUMP-112, CTUMP-113, CTUMP-114, and CTUMP-115 for MO, BJ, EP, ZZ, and CF, respectively) were kept at the Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy. The collected plants were dried, ground, and sieved to appropriate sizes. The RD cell line (ATCC CCL-136^{TM}) was imported from ATCC.

Chemicals for determining plant compositions (i.e.,

diethyl ether, ethanol, and acetic acid) were imported from Xilong, China; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal calf serum (FCS), Eagle's minimum essential medium (EMEM), penicillin-streptomycin (Pen-Strep), trypan blue, trypsin-EDTA, L-glutamine, and dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich, Singapore. The positive control Anzatax® (paclitaxel 30 mg/5 mL) was purchased from Merck, Australia. All other chemicals were of reagent grade or higher.

2.2. Plant extraction

Fresh samples of MO leaves, BJ seeds, EP leaves, ZZ roots, and CF leaves, after being harvested, were washed, sliced, dried at ambient temperature, and finely ground to appropriate sizes. The plant powders with a moisture content of <13% were then extracted and fractionated with three solvents of different polarity, including diethyl ether, water, and ethanol, following the process demonstrated in Figure 1. Briefly, 100 g of the plant powders were macerated with 1200 mL of ether (plant:solvent ratio of 1:12 w/v) for 24 h. The product was divided into two parts: the solution and the plant residues. The solution was condensed with a rotavapor until the moisture content reached <20%, and then used to determine phytochemical compositions and investigate cytotoxicity on cancer cells. The residues were further fractionated with ethanol and water and then divided into two fractions similar to the ether solvent.

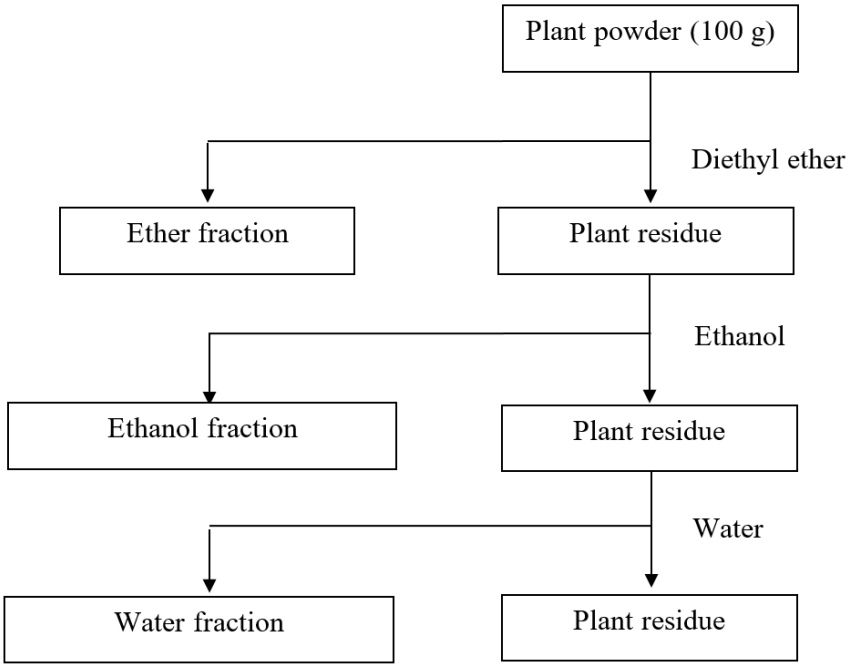

Figure 1. Plant fractionation procedure of 05 selected medicinal plants, including *Moringa oleifera* Lam (MO) leaves, *Brucea javanica* (L.) Merr. (BJ) seeds, *Eclipta prostrata* (L.) (EP) leaves, *Callisia fragrans* (Lindl.) Woodson (CF) leaves, and *Zingiber zerumbet* (L.) Smith (ZZ) roots

2.3. Phytochemical determination

The chemical constituents, in terms of the main compound groups, of the plant fractions (i.e., ether, ethanol, and water) were determined following standard procedures described in Figure 2. Each test was repeated in triplicate to confirm the results.

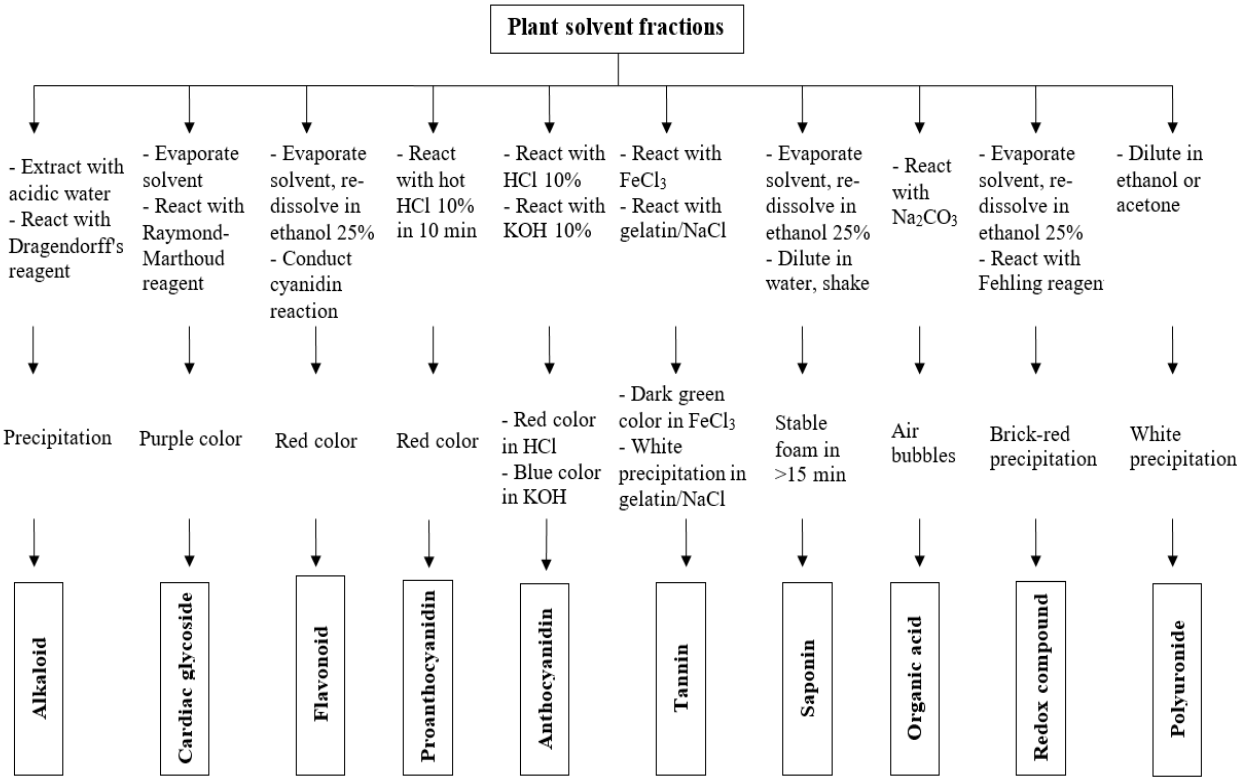

Figure 2. Phytochemical determinations procedures of the fractions (ether, ethanol, and water fractions) of 05 medicinal plants, *Moringa oleifera* Lam, *Brucea javanica* (L.) Merr., *Eclipta prostrata* (L.), *Callisia fragrans* (Lindl.) Woodson, and *Zingiber zerumbet* (L.) Smith.

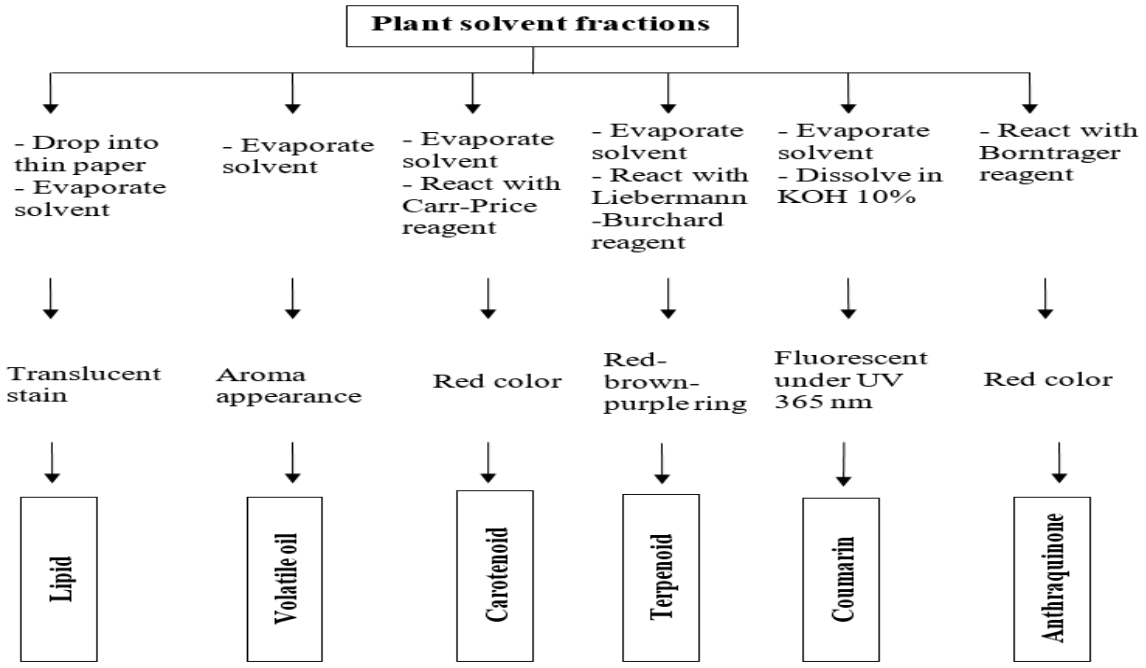

Figure 2 (Continued)

2.4. In-vitro cytotoxicity MTT assay

The cytotoxicity of the plant fractions on RMS was tested using RD cell line. The cells were grown in EMEM medium, supplemented with 10% FCS, 2 mM L-glutamine, and 100 IU/mL + 100 µg/mL PenStrep. Cells were cultured in a 75-cm^2 flask, incubated at 37 ºC with 5% CO_2 in humidified atmosphere, and the medium was changed every even day. Confluent cells (70-80% flask coverage) were washed with PBS, trypsinized with trypsin-EDTA, counted with trypan blue 0.4%, and the cell suspension was transferred into 96-well plates with a density of 12.5 x 10^4 cells/mL (100 µL/well) for the testing experiments.

The plant fraction test samples were prepared in a DMSO solution at an initial concentration of 10 mg/mL and diluted in medium to reach the investigated concentrations of 100, 50, and 10 µg/mL. The negative control was DMSO at the same concentrations in the test samples (1%, 0.5%, and 0.1%, respectively). The positive control was the reference drug paclitaxel. All samples were filtered through a 0.22-µm membrane prior to cell treatments. The samples were subjected to the cells, incubated for 24 h, and the cytotoxic MTT assay was then conducted following the manufacturer's protocol[25,26]. The formed formazan crystal was dissolved in isopropanol, and the solutions were UV-Vis spectroscopically measured at 570 nm with a microplate reader (Multiskan). All experiments were repeated four times. The %Cell inhibitory was calculated based on equation (1).

$$\%\text{Cell inhibitory} = 100\% - \frac{\text{OD sample} - \text{OD blank}}{\text{OD negative control} - \text{OD blank}} \times 100\% \quad (1)$$

2.5. Statistical analysis

The results were processed using Microsoft Excel software and presented as mean ± standard deviation (SD). For statistical significance, the Mann-Whitney test was utilized on SPSS 20.0 software, with $p<0.05$ for meaningful comparisons.

3. RESULTS

3.1. Phytochemical determination

The phytochemical constituents of the five investigated medicinal plants (MO, BJ, EP, CF, and ZZ) are presented in Table 1. Each fraction contained different chemical groups, dependent on their polarity. Overall, the MO leaves contain major components of lipids, carotenoids, volatile oils, flavonoids, tannins, and polyuronides. The BJ seeds mainly possess lipids, alkaloids, coumarins, anthraquinones, and tannins. The EP leaves have volatile oils, terpenoids, alkaloids, flavonoids, saponins, and tannins as the main constituents. The ZZ roots mostly comprise volatile oils, alkaloids, anthraquinones, flavonoids, saponins, tannins, and polyuronides. The CF leaves contain terpenoids, alkaloids, coumarins, flavonoids, proanthocyanidins, saponins, tannins, and polyuronides.

Table 1. Phytochemical constituents of the ether fraction, ethanol (EtOH) fraction, and water fraction of the five investigated medicinal plants, *Moringa oleifera* Lam (MO) leaves, *Brucea javanica* (L.) Merr. (BJ) seeds, *Eclipta prostrata* (L.) (EP) leaves, *Callisia fragrans* (Lindl.) Woodson (CF) leaves, and *Zingiber zerumbet* (L.) Smith (ZZ) roots. (–): absent; (+): present with limited amount; (++): present with moderate amount; (+++): present with high amount

Phytochemical group	*Moringa oleifera*			*Brucea javanica*			*Eclipta prostrata*			*Callisia fragrans*			*Zingiber zerumbet*		
	Ether	EtOH	Water	Ether	EtOH	Water	Ether	EtOH	Water	Ether	EtOH	Water	Ether	EtOH	Water
Lipids	+++	–	–	+++	–	–	–	–	–	+	–	–	+	–	–
Carotenoids	++	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Volatile oils	++	–	–	–	–	–	++	–	–	–	–	–	+++	++	–
Terpenoids	+	–	–	–	–	–	++	–	–	++	–	–	–	–	–
Alkaloids	+	+++	+++	++	+	+	+	+++	+++	++	++	++	++	++	++
Coumarins	–	++	–	–	++	–	–	–	–	++	++	–	–	–	–
Anthraquinones	–	–	–	++	–	–	–	–	–	–	–	–	++	–	–
Flavonoids	–	+++	+++	+	+	+	+	++	++	++	+++	++	++	++	++
Cardiac glycosides	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–
Anthocyanidins	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proanthocyanidins	–	+	+	–	+	–	–	+	+	–	++	++	–	–	–
Tannins	–	+++	+++	–	+++	+++	–	+++	+++	–	++	++	–	+++	+++
Saponins	–	–	–	–	–	–	–	++	++	–	++	++	–	+++	+++
Organic acids	–	++	++	–	+	+	–	++	+	–	++	+	–	+++	++
Redox compounds	–	++	++	–	+	+	–	+	+	–	+	+	–	+++	+++
Polyuronides	–	–	+++	–	–	–	–	–	+	–	–	+++	–	–	+++

3.2. In-vitro cytotoxicity MTT assay

The in-vitro cytotoxicity MTT assay on the RMS cell line RD of the ether fraction, the ethanol fraction, and the water fraction, at concentrations of 100, 50, and 10 µg/mL, of the five medicinal plants (MO, BJ, EP, CF, and ZZ) are presented in Table 2, in comparison with the reference compound, paclitaxel. The results demonstrated that the MO leaves, BJ seeds, and CF leaves did not possess significant cytotoxic effects on the RD cells (i.e., IC_{50} > 100 µg/mL). On the other hand, the EP and ZZ fractions showed high efficacy. Specifically, the EP ether fraction, the ZZ ether fraction, and the ZZ ethanol fraction, had an IC_{50} of 37.08 ± 1.23 µg/mL, 23.15 ± 1.17 µg/mL, and 45.63 ± 2.39 µg/mL, respectively. Compared to the well-known drug paclitaxel with an IC_{50} of 6.96 ± 0.72 µg/mL, these fractions show potential efficacy on the RD cell lines.

Table 2. In-vitro cytotoxicity on the rhabdomyosarcoma RD cell lines of the ether fraction, ethanol (EtOH) fraction, and water fraction, at concentrations of 10, 50, and 100 µg/mL, of the 05 investigated medicinal plants, *Moringa oleifera* Lam (MO) leaves, *Brucea javanica* (L.) Merr. (BJ) seeds, *Eclipta prostrata* (L.) (EP) leaves, *Callisia fragrans* (Lindl.) Woodson (CF) leaves, and *Zingiber zerumbet* (L.) Smith (ZZ) roots. The results are expressed in terms of %Cell inhibitory (mean ± SD) and IC_{50} (mean ± SD) (n = 4). Note: the concentrations of the positive control (paclitaxel) were 10, 5, and 1 µg/mL, correspondingly.

Concentration (µg/mL)	%Cell inhibitory								
	Moringa oleifera			*Brucea javanica*			*Eclipta prostrata*		
	Ether	EtOH	Water	Ether	EtOH	Water	Ether	EtOH	Water
100	3.4 ± 0.5	10.8 ± 1.3	27.8 ± 2.4	14.2 ± 1.3	5.2 ± 0.8	10.5 ± 1.9	**37.8 ± 4.5**	13.6 ± 2.3	0.9 ± 0.2
50	7.3 ± 2.1	7.7 ± 1.8	29.7 ± 3.1	12.8 ± 1.7	13.0 ± 1.1	20.5 ± 2.1	**62.7 ± 4.1**	18.1 ± 3.0	0.2 ± 0.1
10	0.9 ± 0.1	6.3 ± 1.6	20.7 ± 3.4	14.2 ± 2.0	12.6 ± 1.5	12.6 ± 1.4	**31.7 ± 2.8**	18.1 ± 3.1	5.7 ± 1.2
IC_{50} (µg/mL)	> 100	> 100	> 100	> 100	> 100	> 100	**37.08 ± 1.23**	> 100	> 100
Concentration (µg/mL)	*Callisia fragrans*			*Zingiber zerumbet*			*Paclitaxel (concentration)*		
	Ether	**EtOH**	**Water**	**Ether**	**EtOH**	**Water**			
100	29.0 ± 2.5	22.2 ± 3.1	11.3 ± 1.7	**84.2 ± 5.5**	**76.3 ± 7.9**	25.0 ± 1.7	**51.7 ± 5.8 (10 µg/mL)**		
50	27.7 ± 3.4	21.3 ± 3.0	7.8 ± 1.5	**71.9 ± 6.7**	**59.2 ± 4.3**	29.2 ± 3.3	**46.9 ± 4.1 (5 µg/mL)**		
10	19.0 ± 2.8	18.0 ± 2.7	4.1 ± 0.8	**38.7 ± 4.0**	**27.0 ± 3.2**	18.1 ± 2.6	**44.5 ± 4.6 (1 µg/mL)**		
IC_{50} (µg/mL)	> 100	> 100	> 100	**23.15 ± 1.17**	**45.63 ± 2.39**	> 100	**6.96 ± 0.72**		

4. DISCUSSION

RMS, one of the most common cancers in children, has gained increasing attention due to its treatment difficulty[11]. In fact, current RMS treatments involving surgery, radiation therapy, and chemotherapy possess poor outcomes with numerous side effects[7,8]. Thus, novel chemotherapeutic agents are necessary. To this end, this study preliminarily investigated the efficacy of ether, ethanol, and water fractions of five potential medicinal plants, based on local folk remedies in An Giang, Vietnam, in the in-vitro cytotoxicity assay on the RD cell line. Ethanol and water were selected due to folklore wisdom (i.e., these plants are ethnopharmacologically used with ethanol and water as maceration solvents)[19,21,22], whereas ether was chosen because it is a non-polar solvent that could possibly extract the non-polar therapeutic compounds in these plants.

Firstly, the MO plant has been ethnopharmacologically utilized for a long time in An Giang ethnic groups as an antioxidative, anti-inflammatory, antihypertensive, and immuno-regulatory agent. In addition, MO leaves are used in folk remedies for cancer treatments. In terms of phytochemical constituents, the main compounds in MO leaves are lipids, alkaloids, flavonoids, coumarins, and tannins, in agreement with a previous study[27]. Interestingly, the phytochemical compounds in our research were different than the MO leaves in Nigeria[28], which could be due to geographical variations such as soil conditions, weather, and plant growth stages. Regarding its anticancer effect, previous studies have confirmed that the compound niaziminin (a water-soluble thiocarbamate glycoside) in MO leaves possesses high anticancer properties[17,29,30]. Nevertheless, in all three fractions, with different polarities, the MO leaves did not show significant cytotoxicity on the RD cell line, indicating that this plant might not be potential for RMS treatment.

Secondly, the BJ plant is commonly used as food ("goi Sau dau" [Vietnamese] – a Vietnamese salad with a mixture of various vegetables and herbs).

Ethnopharmacologically, the BJ seeds could be ground, and its aqueous extract (i.e., tea) is used to treat diarrhea, appendicitis, and malaria. In BJ seeds, the main components are lipids, terpenoids, alkaloids, flavonoids, saponins, and tannins[31]. Its anticancer property is acknowledged to be based on the compound bruceantin (C28H36O11), a quassinoid. Bruceantin has been proven to have high anticancer activity in various cancers such as lung cancer, myeloma, and gastric cancer[32–35]. Furthermore, in clinical tests with 68 lung cancer patients, BJ seeds extract demonstrates a complementary effect, in conjunction with radiotherapy, in enhancing the patients' quality of life and prolonging their lifespan from 10 months to 15 months compared to radiotherapy alone[32]. However, in our study, the BJ seeds fractions did not show adequate action on the RD cell line.

Thirdly, the EP leaves are popularly utilized to make "tea" by the local people in An Giang. According to folklore, EP tea is believed to possess numerous effects, including antimicrobial, antivirus, pain relief, and anticancer properties. Phytochemically, the EP leaves in An Giang have similar constituents compared to those in other regions[19]. In terms of cytotoxicity activitity, dasyscyphin C ($C_{28}H_{40}O_8$), a saponin in EP, has been confirmed to possess various anticancer effects[36] such as cervical carcinoma (IC_{50} = 50 µg/mL on HeLa cells). Interestingly, in our study, the ethanol and water fractions, which contain lots of saponin, did not yield significant effects on RD cells (IC50 > 100 µg/mL). On the other hand, the ether fraction, with no saponin, exhibited potential cytotoxicity on the RD cell line (IC50 = 37.08 ± 1.23 µg/mL). Further study is necessary to investigate in-depth the biological activities of the components present in the EP leaves ether fraction. Moreover, it is worth noting that the high concentration of 100 µg/mL of the ether extract significantly reduced the EP leaves' cytotoxicity on the RD cells (Table 2). This could be attributed to the fraction's cell proliferation effect. Therefore, the optimal concentration, in this case, was 50 µg/mL. Conclusively, it is necessary to investigate the suitable dose of herbal extracts to ensure their effectiveness in cancer treatment.

Fourthly, the CF leaves are commonly extracted with ethanol to produce a traditional pharmaceutics dosage form called herbal wine. According to the folklore, this wine is a good complementary medicine in treating liver cirrhosis, liver cancer, and other liver-related diseases, as well as acne, joint pain/inflammation, and gout[20]. CF phytochemicals consist of alkaloids, flavonoids, glycosides, coumarins, and saponins, which are in well agreement with previous studies[20,37,38]. In terms of the anticancer effects, to the best of our knowledge, no report (up to 2022) has been published on the CF extract action on the cancerous cells/tissues. Our results, for the first time, showed that CF leaves fractions might not be potent on RD cell line (IC_{50} > 100 µg/mL). Nevertheless, its anticancer effects need further analysis and evaluation on other cell lines, to fill in the literature gap.

Finally, the ZZ roots, which is generally used as “ginger tea” for various therapeutic effects, contain mostly volatile oils, flavonoids, saponins, and alkaloids, which are in correlation with previous work[39]. Among these chemicals, zerumbone ($C_{15}H_{22}O$), a sesquiterpene volatile oil with a humulan-based carbon framework, possesses outstanding anticancer activity[40]. For example, the anticancer activity of zerumbone against the human HeLa cell line was confirmed with an IC_{50} of 2.5 µg/mL[41]. In our work, the IC_{50} values of the ether fraction and ethanol fraction of ZZ roots were 23.15 µg/mL and 45.63 µg/mL, respectively, indicating the potential effects of this plant on RMS cell line RD. The fact that the ether fraction was more potent than the ethanol fraction could be contributed to the higher amount of volative oils (i.e., zerumbone) in the former (Table 1)[42,43].

5. CONCLUSION

This study investigated the potency of five common medicinal plants (MO, BJ, EP, CF, and ZZ) in An Giang, Vietnam, for the treatment of RMS using RD cell line. The EP ether fraction, the ZZ ether fraction, and the GIN

ethanol fraction, possess moderate cytotoxic effects on RD cell lines, with an IC_{50} of 37.08 ± 1.23 µg/mL, 23.15 ± 1.17 µg/mL, and 45.63 ± 2.39 µg/mL, respectively. These results provide preliminary data for further in-depth research on the RMS anticancer properties of these plants, especially the EP and ZZ plants, which are widely grown in the South of Vietnam.

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Conflict of interest

None to declare.

Ethical Approval

Ethical Approval is not applicable for this article.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

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النباتات الطبية المحلية (Moringa oleifera Lam)، Brucea javanica (L.) Merr.، Eclipta prostrata (L.)، Callisia fragrans (Lindl.) Woodson، و Zingiber zerumbet (L.) Smith)في آن جيانج، فيتنام: تحقيق أولي لعلاج الساركوما العضلية المخططة باستخدام اختبار السمية الخلوية لخلايا RD في المختبر

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ملخص

يُقدر أن السرطان، وهو أحد أكثر الأمراض فتكًا في جميع أنحاء العالم، سيؤثر على 30.2 مليون شخص بحلول عام 2040. ومن بين أكثر من 100 نوع من السرطان، يعد الساركوما العضلية المخططة (RMS)نوعًا خاصًا من الأورام التي تؤثر في الغالب على الجهاز العضلي للأطفال. يمتلك علاج RMSالحالي فعالية محدودة والعديد من الآثار الجانبية. وبالتالي، فإن العلاجات الجديدة ضرورية. هنا، بحثت هذه الدراسة في إمكانات علاج خط خلايا RMS RDلخمسة نباتات طبية قائمة على الفولكلور في آن جيانج، فيتنام. تم استخلاص النباتات (Moringa oleifera Lam)، Brucea javanica (L.) Merr.، Eclipta prostrata (L.)، Callisia fragrans (Lindl.) Woodson، و Zingiber zerumbet (L.) Smith)وتفتيتها في ثلاثة مذيبات من الأثير، تم فحص الإيثانول والماء، وتم فحص هذه الأجزاء الكيميائية النباتية واختبار السمية الخلوية في خط خلايا RMSفي المختبر .RD.أظهرت النتائج أن جزء إيثر Eclipta prostrata (L.) وجزء Zingiber zerumbet (L. Smith ether) وجزء الإيثانول، لهما تأثيرات سامة للخلايا معتدلة على خطوط خلايا RD، مع IC50 يبلغ 37.08 ± 1.23 ميكروغرام / مل، 23.15 ± 1.17 ميكروغرام/مل، و45.63 ± 2.39 ميكروغرام/مل، على التوالي. توفر هذه النتائج بيانات أولية لإجراء مزيد من الأبحاث المتعمقة حول خصائص RMSالمضادة للسرطان لهذه النباتات، والتي تنمو بشكل كبير في جنوب فيتنام.

الكلمات الدالة: الساركومة العضلية المخططة. المورينغا أوليفيرا لام؛ بروسيا جافانيكا (.L) مير ؛ إكليبتا بروستراتا (.L)؛ عطر كاليسيا (ليندل) وودسون؛ زينجيبر زيروميبت (.L) سميث؛ السمية الخلوية. تجزئة.

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Knowledge, Willingness to Pay and Beliefs for Seasonal Influenza Vaccination, A Cross-Sectional Study from Jordan

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ABSTRACT

Background: Seasonal influenza is a viral illness associated with significant morbidity and mortality.
Objectives: This study aimed to assess preferences for utilizing the seasonal influenza vaccine.
Methods: Based on a review of the literature and discussions among the research team, a 37-item survey was created, pretested, and completed by the lay public in Irbid city. The survey assessed knowledge, willingness-to-pay, and beliefs regarding the seasonal influenza vaccine. Participants' willingness-to-pay for the influenza vaccine was determined using contingent valuation with a payment card. Logistic regression analysis was employed to determine predictors associated with willingness-to-pay.
Results: A total of 347 responses constituted the study sample. Respondents rated their knowledge about the influenza vaccine as good or excellent (62.5% of the total received responses). Approximately one-half (45.3%) of the respondents were willing to pay 5 JD for the influenza vaccine. It was found that standard of living, living location (city vs. village), and occupation (i.e., employment status) were independent predictors associated with higher willingness-to-pay for the influenza vaccine.
Conclusion: The participants were willing to pay a price close to the market price, thus affording the vaccine. Such data can help healthcare decision-makers develop promotive policies to improve vaccine uptake.
Keywords: Seasonal influenza vaccine, knowledge, willingness to pay, Jordan.

INTRODUCTION

Influenza is a viral illness associated with significant morbidity and mortality, making it a public health problem (1). Every year, seasonal influenza imposes substantial health and economic burdens on society. The influenza vaccine has been employed to reduce these morbidities and mortalities, particularly in elderly patients. Consequently, the Centers for Disease Control and Prevention (CDC) recommend vaccination for the elderly as a risk-reduction strategy against contracting influenza and its complications (2, 3). To address the impact of influenza on workdays, associated costs, and other issues, in the United States, free influenza vaccines have been offered to employees by their employers. It is considered one of the standard vaccines for healthcare workers, and their vaccine acceptance is based on perceived effectiveness, perceived likelihood of side effects, and whether they received the vaccine the previous year (4).

In the development of seasonal influenza vaccines, alterations in the influenza strains are usually carried out to target the strains expected to be present in upcoming seasons (5). Despite the anticipated challenges of

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influenza vaccination, only a high level of quality evidence has been found for these vaccines in the prevention of laboratory-confirmed influenza. Considerably limited evidence exists for the expected benefits of the influenza vaccine in the presence of complications (e.g., pneumonia) (6). Overall, influenza vaccines are considered the best method for preventing and controlling influenza due to their safety and effectiveness profile. In terms of cost-effectiveness, studies have found influenza vaccination to be cost-effective, and even a cost-saving approach (7).

To date, the influenza vaccine has not been included in medical insurance schemes in Jordan. Influenza has been associated with a significant burden on the healthcare system. The present study aimed to explore public preferences for utilizing the influenza vaccine, with a specific focus on a willingness-to-pay approach. Willingness-to-pay is defined as "the maximum amount that people are willing to pay to gain outcomes that they view as desirable," in this case, the purchase of an influenza vaccine (8). Additionally, the study aimed to assess the beliefs of respondents surrounding the utilization of the influenza vaccine and identify predictors that might influence public willingness to pay for the influenza vaccine.

METHODOLOGY

Using survey methodology, insights into public preferences toward the influenza vaccine were obtained. A purposive questionnaire was distributed by hand to the lay public recruited from the city of Irbid in northern Jordan over the period November 2019 to January 2020. The survey was distributed in areas and shops around the university district in Irbid by two trained students.

Survey Design

A 37-item instrument was developed to gather information about background and demographic characteristics, knowledge, behaviors to deal with influenza disease, consequences while getting the disease, willingness to pay for the influenza vaccine, willingness to pay for specific desirable beneficial outcomes associated with vaccine use (e.g., not missing a day from work), and respondents' beliefs regarding vaccination.

The survey design was constructed based on a literature review of published work in the field (9, 10). Content and face validity were confirmed based on comments from faculty members with experience in this type of research. A content validity index was calculated using input from six faculty members within the School of Pharmacy at Yarmouk University holding MSc or Ph.D. degrees. The content validity index (CVI) measures the agreement between independent raters for the validity of items, with the item-CVI used as a measure of agreement for each item. The average of the item-CVI was used to assess the overall scale-CVI. It is recommended that the scale-CVI be 0.83 or higher, using input from six experts (11). The average content validity index was 0.915, and based on these assessments, modifications to the survey instruments were carried out.

Pilot exercise was carried out by distributing the survey to ten individuals, during which the clarity of questions and the logistics of distribution were assessed. The pilot data were included in the final study, and minor modifications were made whenever needed based on the pilot distribution results. With a Cronbach's alpha of 0.780, the internal consistency of the present study instrument was considered acceptable (12). For individual constructs such as the willingness to pay component, the Cronbach’s alpha was 0.858, and for the beliefs about the influenza vaccine section, it equaled 0.846.

The target population was current residents of Irbid city in Northern Jordan who are 18 years old. The survey

was anonymous to ensure respondents' confidentiality and privacy. Consent to take part in the study was considered as granted from the respondent by their agreement to sign the consent form. Ethical approval was obtained from the Institutional Review Board (IRB) at Jordan University of Science and Technology, Irbid (8/127/2019). Based on the population of adults in Irbid, with a margin of error of 5.3% and a 95% confidence level, the sample size calculation using an online sample size calculator, Raosoft (available at http://www.raosoft.com/samplesize.html), revealed a minimum sample size of 342 responses. This figure is similar to other recent surveys carried out (13-15).

Willingness to Pay

Respondents were asked to indicate their willingness to pay (WTP) for a self-paid influenza vaccine using a contingent valuation method. Before deciding on the best WTP value using the payment card approach, respondents were given the following informative statement: "The influenza vaccine has been demonstrated to minimize flu-related symptoms as well as the risk of significant flu complications, which can result in hospitalization or even death. Annual influenza vaccination is recommended, especially for high-risk groups, such as persons 65 and older, people with certain chronic medical conditions (such as asthma, diabetes, or heart disease), pregnant women, and children under the age of five, etc." Respondents were not informed of market pricing; instead, they were asked to choose their WTP from a range of values that included market prices. The choices provided for the participants were close-ended and were JD0, JD5, JD15, and JD25.

Data analysis

All collected data were entered using Microsoft Excel, and then the statistical analysis was carried out using IBM® SPSS® Statistics V25.0 (Armonk, NY, USA: IBM Corp.). Descriptive statistics (means and frequencies) were used for data description. Logistic regression (binary and ordinal) was used to assess the factors that affect the willingness to pay by the participants. Statistical significance was set to $p = 0.05$.

Results

Results of the current study were obtained from 347 received responses out of a total of 450 surveys distributed to the public in Irbid city, Jordan (a response rate of 77.1%). The sample was characterized by a higher proportion of females (62.0%) compared to males (38.0%). The majority (84.0%) of the respondents were in the age range of 18 to 29 years old, and 89.6% of the respondents were single. Unemployed individuals and students together constituted 72% of the sample. Most respondents (76.9%) reported an intermediate standard of living. A total of 55.3% of respondents lived in the city, while 44.7% lived in rural areas. Among the entire sample, 82.7% had health insurance. The full details regarding the demographics of the respondents are summarized in Table 1.

Table 1 Frequency distribution for the sociodemographic factors (N= 347)

Variable	Frequency (%)
Gender	
Male	132(38)
Female	215(62)
Age	
18 – 24 years	168(48.4)
25 – 34 years	143(41.2)
35 or above	36(10.4)
Education	
Secondary school or lower level	43(12.4)
Bachelor's degree	271(78.1)
Higher education	33(9.5)
Marital Status	
Single	261(75.2)
Married	86(24.8)
Occupation	
Unemployed	90(25.9)
Medical occupation	17(4.9)
Nonmedical occupation	80(23.1)
Student	160(46.1)
Standard of living	
Low	53(15.3)
Intermediate	267(76.9)
High	27(7.8)
Residence area	
Rural	155(44.7)
City	192(55.3)
Insurance	
Not insured	60(17.3)
Insured	287(82.7)
Perceived Knowledge of Flu Vaccine	
Fair	132(38)
Good	170(49)
Excellent	45(13)

Figure 1 illustrates the disease status of respondents. Approximately half (46.7%) of the respondents reported no diseases, while less than 10% suffered from conditions such as asthma, lung problems, sinusitis, or ear problems. Additionally, 3.5% of the respondents had heart disease, and another 3.5% had diabetes mellitus. A total of 8.4% of the respondents reported being smokers.

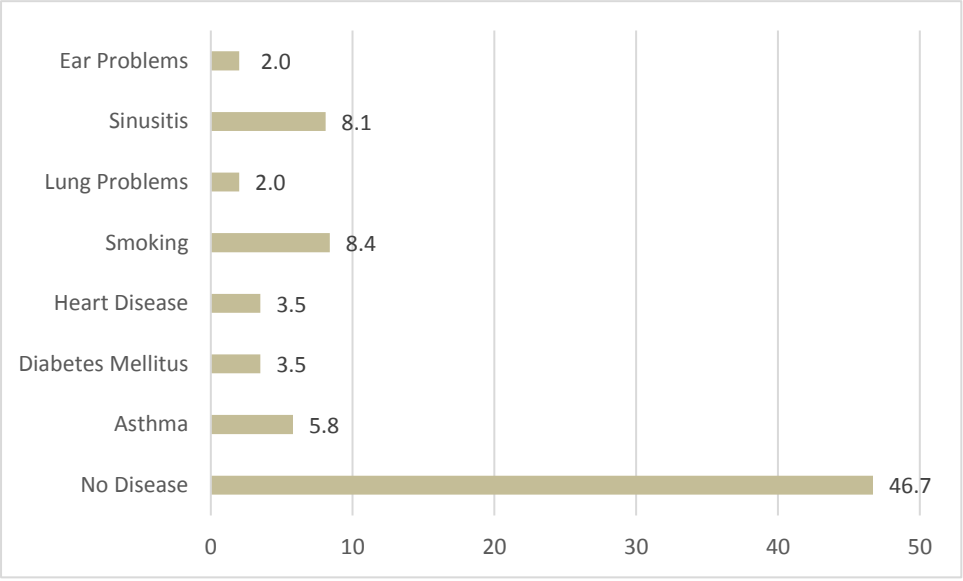

Figure 1: Disease present in the study sample

Table 2 provides details on the assessment of general knowledge about the influenza vaccine among the respondents and its relationship to willingness to pay. The respondents rated their knowledge of the influenza vaccine as good or excellent, accounting for 62.5% of the total respondents. Approximately 44.7% of respondents consulted a medical doctor if they had influenza, while 27.7% consulted a pharmacist. About 58.8% of the respondents believed that the influenza vaccine should be taken annually, and 59.7% thought that influenza virus strains change their shape every season. Independent predictors associated with the willingness to pay for the influenza vaccine were those who consulted a pharmacist (odds ratio = 3.676; p-value = 0.002) and those with the correct knowledge that the influenza vaccine should be taken annually (1.853; 0.017).

Table 2 Logistic regression for the association between willingness to pay and general knowledge of the Flu vaccine

Variable	Frequency (%)	Willing vs Not-Willing[1]	
		OR (95% CI)	P-value
Knowledge of influenza vaccine			
Fair	132(38)	1	
Good	170(49)	1.075(0.629-1.835)	0.791
Excellent	45(13)	1.28(0.557-2.941)	0.561
What to do if you had influenza			
Do nothing	52(15)	1	
Ask lay person	25(7.2)	1.823(0.621-5.355)	0.275
Consult medical doctor	155(44.7)	1.773(0.905-3.474)	0.095
Consult pharmacist	96(27.7)	3.676(1.631-8.285)	0.002
Use information from media and Internet	19(5.5)	1.871(0.534-6.562)	0.328
Do you think that influenza vaccine should be taken annually			
No	143(41.2)	1	
Yes	204(58.8)	1.853(1.119-3.069)	0.017
Do you think that influenza virus strains change its shape at every season			
No	140(40.3)	1	
Yes	207(59.7)	1.168(0.698-1.953)	0.554

[1] those who selected WTP as zero or not

Figure 2 illustrates the willingness to pay (WTP) for the influenza vaccine among respondents. Approximately one quarter (23.5%) are not willing to pay anything for influenza vaccination. About half (45.3%) of the respondents were willing to pay 5 JD for influenza vaccination. A total of 23.4% of the respondents were willing to pay 15 JD for influenza vaccination, and 7.9% were willing to pay 25 JD for influenza vaccination.

Table 3 presents the logistic regression analysis for the association between willingness to pay and demographic factors. It was found that a higher educational level (odds ratio = 5.205; p-value = 0.002), a higher living standard level (odds ratio = 4.408, p-value = 0.015), and living in the city as opposed to a rural area (odds ratio = 1.835, p-value = 0.018) were independent predictors for willingness to pay for seasonal influenza vaccination.

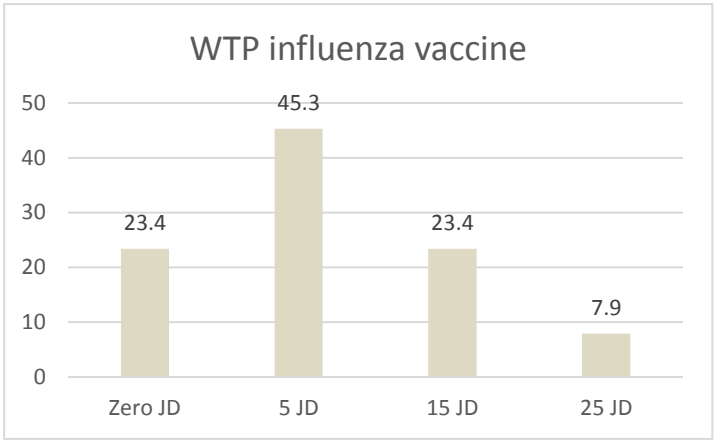

Figure 2: WTP influenza vaccine

Table 3 Logistic regression for the association between willingness to pay and other factors

Variables	Willing vs Not-Willing[1]	
	OR (95% CI)	**P-value**
Gender		
Male	1	
Female	1.191(0.716-1.984)	0.501
Age		
18 – 24 years	1	
25 – 34 years	0.92(0.484-1.748)	0.799
35 or above	1.222(0.679-2.199)	0.504
Education		
Secondary school or lower level	1	
Bachelor's degree	4.963(2.665-9.242)	0.00
Higher education	5.205(1.861-14.557)	0.002
Marital Status		
Single	1	
Married	1.397(0.756-2.584)	0.286
Occupation		
Unemployed	1	
Medical occupation	2.703(1.272-5.743)	0.01
Nonmedical occupation	1.365(0.617-3.02)	0.442
Student	1.055(0.581-1.914)	0.86
Standard of living		
Low	1	
Intermediate	3.096(1.664-5.76)	0
High	4.408(1.338-14.529)	0.015
Residence area		
Rural	1	
City	1.835(1.108-3.039)	0.018
Insurance		
Not insured	1	
Insured	0.903(0.509-1.602)	0.727

[1] those who selected WTP as zero or not

The assessment of beneficial outcomes associated with the use of influenza vaccination and the respondents' willingness to pay for these aspects was summarized in Table 4. The most commonly reported willingness to pay (WTP) was 5 JD for various aspects related to influenza, such as avoiding symptoms, tiredness associated with influenza, and not missing days from work. The least commonly reported WTPs were 0 JD (nothing) and 1 JD for many of the aspects. A significant proportion of respondents were willing to pay higher amounts (25 and 15 JD) for many aspects.

Table 4. Percentage of WTP to different beneficial outcomes associated with the use of influenza vaccine

Aspect/WTP	0 JD	1JD	5 JD	15JD	25JD
Not to suffer from the symptoms and tiredness associated with influenza	12	9	38.5	17.5	23
Not to miss one day from work	25.7	8.5	37.4	17.5	10.8
Not to suffer from the fever and joint pain associated with influenza	7.6	11.7	30.5	24	26.1
To take the vaccine once yearly	10.9	12.6	34.7	27.1	14.7
Not to take the medications for influenza	14.2	12.4	34.3	23.4	15.7
To maintain the health-related quality of life	8.5	9.7	26.1	19.9	35.8

Table 5 summarizes the respondents' beliefs regarding the influenza vaccine. The table presents the degree of agreement with different statements that indicate positive beliefs about vaccines in general. A significant percentage of respondents either agreed or strongly agreed with various positive belief statements. For instance, 53.4% of the respondents agreed or strongly agreed with the statement "taking the influenza vaccine is the right thing to do," and 66.6% of the respondents agreed or strongly agreed with the statement "vaccines are important to maintain health."

Table 6 illustrates that marital status (specifically, being married) and higher age are factors associated with the summated score for beliefs regarding the influenza vaccine, as determined through univariable linear regression analysis.

Table 5 Frequency of respondents' beliefs regarding influenza vaccine (N=347)

	Disagree (%)	Neutral (%)	Agree (%)	Strongly agree (%)
Taking influenza vaccine is the right thing to do	20.1	26.5	11.4	42
Vaccines are important to maintain health	7.6	25.9	19.8	46.8
it is important to take vaccine to prevent spread if infections	6.1	15.7	23	55.2
Influenza Vaccines are safe	8.5	27.2	24.9	39.5
if vaccines are not taken, disease can be spread to other individuals	13.2	21.6	25.4	39.8
We can develop serious disease if we don't take vaccines	19.8	24.5	18.1	37.6

Table 6 factors associated with summated score for beliefs regarding influenza vaccine using univariable linear regression analysis

	Beta coefficient (Standard error)	P value	95.0% Confidence Interval for B
Marital status, married	-1.766 (0.568)	0.002	-2.883 – -.648
Residence area	0.986 (0.503)	0.051	-.004 -1.977
Age, higher age	-1.309 (0.464)	0.005	-2.223 - -.395
WTP	0.460 (0.286)	0.109	-.103 - 1.023
Job new	-1.179 (0.627)	0.061	-2.413 - 055

The predictors of the multivariate ordinal logistic regression model for willingness to pay (WTP) for influenza vaccines are summarized in Table 7. According to the multivariate ordinal logistic regression model, it was found that standard of living, living location (city vs. village), and occupation (specifically, being employed) were independent predictors associated with WTP for influenza vaccine.

Table 7 Ordinal logistic regression for the factors associated with willingness to pay for influenza vaccine

Variable		WTP (0,5,15,25 JD)		
		Odds ratios	P value	95% confidence interval of the odds ratio
Education	Secondary school or lower level	1.95	0.432	0.37 - 10.23
	Bachelor's degree	1.47	0.521	0.45 - 4.75
	Higher education	Reference		
Gender	Male	1.09	0.760	0.63 - 1.89
	Female	Reference		
Living standard	Low	0.21	0.008	0.07 - 0.67
	Intermediate	0.51	0.167	0.20 -1.32
	High	Reference		
Living location	City	1.68	0.046	1.01 -2.79
	Village	Reference		
Health insurance	Insured	1.23	0.530	0.64 - 2.38
	Uninsured	Reference		
What to do if you had influenza	Do nothing	1.06	0.926	0.32 - 3.52
	Ask lay person	1.66	0.506	0.37 - 7.46
	Consult doctor	1.48	0.487	0.49 -4.41
	Consult pharmacist	1.62	0.423	0.50 - 5.28
	Media and internet	Reference		
Age (years)	18-29	3.51	0.075	0.88 - 13.99
	30-39	1.73	0.468	0.39 - 7.68
	40-65	Reference		
Occupation	Unemployed	0.41	0.037	0.18 - 0.95
	Employed	Reference		

DISCUSSION

Seasonal influenza imposes a significant burden on the healthcare system, particularly affecting the elderly, and influenza vaccination is considered the most cost-effective approach to prevent its impact (16-18). This study aimed to assess the willingness to pay (WTP) for influenza vaccine among the lay public, their willingness to pay for specific desirable outcomes associated with vaccine use, and the factors influencing WTP for influenza vaccine. The findings indicate that approximately 75% of the sample is willing to pay JD5 or more for the influenza vaccine. Respondents with a high and intermediate standard of living, as well as those who consult pharmacists, were identified as independent predictors for WTP for influenza vaccine through multivariate ordinal logistic regression analysis. The amount participants are willing to pay for the influenza vaccine in this study is similar to or higher than the market price for the vaccine. Access to pharmacists, the most accessible healthcare professionals, is posited to facilitate vaccine accessibility, and consulting with pharmacists independently predicts the willingness to pay for influenza vaccine. It is noteworthy that the administration of vaccines in community pharmacies has been legislated in Jordan (19).

The WTP was examined in this study using a payment card approach, encompassing not only the amount participants were willing to pay for the vaccine but also various desired outcomes related to its administration, such as how much they were willing to pay to avoid influenza symptoms and not miss a day of work. The majority were willing to pay the amount of JD5, signifying positive news as the overwhelming majority indicated a willingness to pay from JD5 to JD25. Research conducted in China, using a bidding game to estimate the WTP for seasonal influenza vaccine, found a median WTP of 10 US dollars, with 45% of the sample willing to pay the market price for the vaccine (20). Another Chinese study found an average WTP of 13.7 US dollars for respondents with chronic diseases and 12.5 US dollars for the elderly (21). Another group highlighted a vaccination rate of 54.3% for age groups 50 years and older (22). The encouraging WTP results suggest that price may not be a major hindrance, indicating a potential for high vaccine uptake. Despite this, the vaccination rate in Jordan was estimated to be between 9.9% and 27.5% (23). Another study from Jordan found a low vaccination rate (20%) (24).

The cost of preventive measures, such as vaccines, from an economic perspective, involves current expenditures to avert delayed problems or gain benefits (4). An overarching factor that could impact the willingness to pay (WTP) results in the present study is the presence of a substantial proportion (about three-quarters) of respondents who were unemployed or students, and approximately ten percent of respondents had a low standard of living. For individuals to afford the vaccine, the vaccine price should be perceived as low, and the user's ability to pay should be high (20).

Contact with formal healthcare systems is crucial for more serious diseases; however, disease and treatment knowledge, along with health literacy, are critical components that enable patients to derive the most benefit from consultations. The present study assessed the knowledge respondents had regarding influenza and influenza vaccine. Knowledge is an essential prerequisite for the uptake of the seasonal influenza vaccine and was identified as an independent predictor for the willingness to receive the influenza vaccine in the future (25, 26). In a study surveying healthcare workers, basic influenza knowledge was more associated with the group of healthcare workers who were vaccinated, suggesting that low knowledge of vaccination can impede vaccination rates even among healthcare providers, serving as a barrier (23). A questionnaire-based study in Jordan found that about half of the respondents were considered knowledgeable, with low knowledge regarding the role of seasonal influenza vaccination in disease prevention. This highlights a potential impact on vaccination coverage (27).

Almost half of the respondents reported that the initial point of contact for influenza would be a physician, and

about one-quarter would consult a pharmacist. The provision of influenza vaccination within community pharmacies aims to increase vaccination access and overall uptake. A study in the USA found that, despite pharmacists providing influenza vaccinations to millions of individuals, this did not significantly increase the overall vaccination rate. The direct benefit of such an approach would be to provide a more convenient way to administer the vaccine along with increased services from pharmacists (28). It is encouraging that a low percentage of respondents sought advice from laypersons like friends and used information from the media and the internet. Both are external factors that could influence the utilization of medicine and affect patient beliefs. However, the low quality of advice from laypersons and the noted potential influence of media and internet information on individual health decisions should be considered (29, 30).

Risks associated with vaccines have received considerable media attention, especially concerning childhood vaccines and the recent COVID-19 vaccine. Such issues can have negative consequences for the health of individuals and society overall. Respondents in this study expressed beliefs that vaccination is the right thing to do, vaccines are important for maintaining health, and it is crucial to take the vaccine to prevent the spread of disease. Such positive beliefs can contribute to increased influenza vaccine uptake. Occasionally, unhelpful attitudes may be expressed; for example, some elderly individuals may perceive themselves as not at risk of death from influenza. Despite this, a study conducted in the UK found that the elderly might choose to take the vaccine, and their attitudes are generally in favor (31). A UK study that assessed the uptake of influenza vaccine, knowledge about the vaccine, attitudes, and factors associated with seasonal influenza vaccine utilization among healthcare workers using an online survey found a positive attitude toward the vaccine (32). At the country level, vaccine uptake appears to be higher in countries with normative beliefs about vaccination use, including the degree to which important people to them believe they should or should not engage in vaccination behavior (33). Those with a positive attitude toward vaccination are more likely to accept the vaccine (34).

It has been noted that a higher standard of living is an independent predictor of willingness to pay (WTP) for the influenza vaccine. Patients with a higher standard of living are generally in a better societal position. Higher access to care is influenced by socioeconomic factors, particularly economic barriers to vaccination (20). Health beliefs and other vaccine-related aspects can be associated with higher WTP for seasonal influenza vaccine, including media coverage of deaths, the associated costs, and the perceived importance, safety, and efficacy of the vaccine (4, 20, 21, 33, 35). A study from Jordan found that the perceived risk of the vaccine and higher perceived benefits are important factors affecting the WTP for the COVID-19 vaccine (36). In the present study, knowledge about the seasonal influenza vaccine was associated with WTP for the vaccines. Other vaccine-related research conducted in Jordan highlighted that a major factor for not accepting the human papillomavirus vaccine is the low perceived risk of getting an infection (37). Another study from Jordan assessed factors affecting the utilization of the influenza vaccine and found that most respondents are willing to receive the influenza vaccine if provided free of charge (38).

Several future research ideas can be proposed to address contextual issues related to seasonal influenza and influenza vaccination. These include efforts to change societal attitudes and better inform the public about the value of vaccinations, assessing the impact of the COVID-19 pandemic on vaccination rates, and developing novel vaccine formulations that offer improved properties while retaining safety and efficacy.

There were a few limitations that affect the interpretation of the current research findings, including range bias, wherein the respondent's choice of the maximum WTP depends on the range of values offered by the payment card

(39). Despite the potential for such bias, the payment card's value range was carefully chosen to encompass the market price, as well as values both above and below it. Another limitation that restricts the generalizability of the study is the large proportion of unemployed, student, and young respondents, which influenced the amount that respondents were willing to pay.

CONCLUSION

The study findings revealed that the majority of participants were willing to pay for annual influenza vaccines, with amounts ranging from 5 JD to 25 JD, indicating the perceived benefits of the vaccine. Several factors highlighted in the present study significantly influenced consumer decisions to pay for the vaccine, particularly consulting pharmacists, educational level, living standard, and residing in urban areas. A larger and more representative study is needed to investigate other factors that might contribute to consumers' decisions. While almost one-fourth of the sample of commuters preferred not to pay for the vaccine, such data could help healthcare policymakers and decision-makers develop promotive policies to improve vaccination uptake and increase its accessibility.

Conflicts of Interest: None to declare.

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المعرفة والاستعداد للدفع والمعتقدات الخاصة بالتطعيم ضد الأنفلونزا الموسمية، دراسة مقطعية من الأردن

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ملخص

الخلفية: الأنفلونزا الموسمية مرض فيروسي مرتبط بمعدلات امراض ووفيات كبيرة.

الأهداف: هدفت هذه الدراسة إلى تقييم الأفضليات لاستخدام لقاح الأنفلونزا الموسمية.

الطرق: استنادا إلى مراجعة الأبحاث المنشورة والمناقشة بين فريق البحث، تم تطوير استبيان مكون من 37 بند، وتم اختباره مسبقا، وتمت تعبئته من قبل العامة في مدينة إربد. يقيم الاستبيان المعرفة والاستعداد للدفع والمعتقدات المتعلقة بلقاح الأنفلونزا الموسمية. تم تحديد مدى استعداد المشاركين لدفع ثمن لقاح الأنفلونزا باستخدام بطاقة الدفع. تم استخدام تحليل الانحدار اللوجستي لتحديد العوامل المرتبطة بالاستعداد للدفع.

النتائج: بلغ عدد إجابة عينة الدراسة 347 إجابة. صنف المشاركون معرفتهم بلقاح الأنفلونزا بأنها جيدة أو ممتازة (62.5% من إجمالي الاستجابات). أبدى ما يقرب من نصف (45.3%) من أفراد العينة استعدادهم لدفع 5 دنانير مقابل لقاح الأنفلونزا. لقد وجد أن مستوى المعيشة، وموقع المعيشة (المدينة مقابل القرية) والمهنة (الموظفون) كعوامل مرتبطة بارتفاع الرغبة في الدفع للقاح الأنفلونزا.

الاستنتاج: كان المشاركون على استعداد لدفع سعر قريب من سعر السوق، وبالتالي لديهم القدرة للحصول على اللقاح. يمكن أن تساعد مثل هذه البيانات صناع القرار في مجال الرعاية الصحية على تطوير سياسات ترويجية لاستخدام اللقاح.

الكلمات الدالة: لقاح الأنفلونزا الموسمية، المعرفة، الاستعداد للدفع، الأردن.

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Physicians' Knowledge of Theophylline Use: A Cross-Sectional Study from Jordan

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ABSTRACT

Objective: This cross-sectional study aims to assess physicians' knowledge regarding theophylline drug and other related characteristics in Jordan.

Materials and Method: The study was conducted prospectively among physicians in Jordan. Physicians were interviewed using an online questionnaire consisting of two sections. The first section included demographics and other relevant characteristics, while the second section comprised questions about theophylline drug.

Results: A total of 385 participants completed the questionnaire. The majority of participants knew that theophylline is used in clinical practice as a bronchodilator (75.6%). Nearly 39% of participants knew that theophylline can be administered orally and intravenously. The largest share of participants (76.1%) did not know that theophylline dosage is calculated based on ideal body weight. Sixty percent of participants knew that theophylline use was not contraindicated during pregnancy. On the other hand, only 27.3% knew that theophylline use was not contraindicated during breastfeeding. The majority of participants (76.1%) had an overall intermediate knowledge of theophylline.

Conclusions: It was noted that physicians had an overall intermediate knowledge of theophylline. Physicians demonstrated unsatisfactory knowledge about theophylline's indications, clinical use, administration, adverse effects, and other related aspects. These findings highlight the need for educational interventions and training programs to improve physicians' knowledge of theophylline and enhance its effective and safe use in clinical practice.

Keywords: Physicians; Knowledge; Theophylline; Jordan.

INTRODUCTION

Theophylline has been used in the treatment of airway diseases for over 80 years and remains one of the most widely prescribed drugs due to its cost-effectiveness and widespread availability (1, 2). Theophylline is a bronchodilator indicated for managing bronchospasm in respiratory diseases like asthma and chronic obstructive pulmonary disease (COPD) (1, 3). It effectively treats and prevents bronchospasm-induced symptoms such as shortness of breath, wheezing, and chest tightness (1). Theophylline exerts its bronchodilatory effect by competitively inhibiting type III and type IV phosphodiesterase (PDE) enzymes (1, 4). It also has anti-inflammatory action by inhibiting PDE4 and activating histone deacetylase-2 (1, 4).

In clinical practice, theophylline can be used as an

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alternative therapy in the treatment of persistent asthma in combination with other asthma medications, such as inhaled corticosteroids and bronchodilators (5, 6). For COPD treatment, theophylline may be considered in patients who are intolerant of or unable to use inhaled bronchodilators (1). It can also be used as add-on therapy to the regimen of patients whose COPD is not controlled despite the use of inhaled bronchodilators (1).

Theophylline can be administered orally as extended-release tablets; capsules; and elixirs and as an intravenous solution (7). Aminophylline is an ethylenediamine salt of theophylline that has the same indications as theophylline (6). However, it is less potent and has a shorter duration of action than theophylline (1). In addition to its efficacy, theophylline is inexpensive and widely available (3). However, its use is limited by its narrow therapeutic index and multiple drug-drug interactions (1, 8, 9). Further, the serum theophylline concentrations require close monitoring (1, 10). Therapeutic concentrations for theophylline in adults should be maintained between 10-20 mcg/ml. Theophylline toxicity occurs when theophylline's serum concentration is above 20 mcg/mL and is manifested by gastrointestinal distress, insomnia, and tremor (1, 11). Moreover, theophylline may cause serious side effects including arrhythmias, convulsions, and seizures, and may lead to death (1, 12). According to the Association of Poison Control Centres (AAPCC), there were 2 deaths out of 81 patients due to theophylline toxicity (13).

Although theophylline use is limited nowadays, the most recent guidelines about the management of asthma and COPD, still list theophylline as a third or fourth-line therapy (2, 6, 14, 15). Additionally, interest in its use for the treatment of poorly controlled patients is resurging (8, 16-18). Due to several restrictions on theophylline use, physicians prescribing this drug should have an appropriate level of knowledge regarding theophylline's efficacy and safety to optimize treatment while avoiding adverse effects and toxicity (19, 20).

To the best of the researchers' knowledge, there are no related studies that have assessed the level of knowledge among physicians regarding theophylline use in Jordan. Furthermore, studies on the prescribing patterns of theophylline by physicians in Jordan are also lacking. However, strong evidence from several studies reveals a high prevalence of prescribing errors among physicians in Jordan, with most of these errors being clinically significant. These errors frequently include drug-drug interactions, inappropriate doses, wrong dosage forms, unnecessary drug therapy, and monitoring parameters, and theophylline was one of the treatments mentioned in these studies (21, 22).

Despite the absence of published data about theophylline prescribing patterns and use in Jordan, conducting this study addresses an essential gap in the literature and provides insights into theophylline management in the Jordanian healthcare system. The findings of this study can establish the basis for educational initiatives, empowering physicians with the necessary knowledge to make well-informed clinical decisions that ultimately enhance patient care and prevent potential toxicity. Additionally, enhancing physicians' knowledge of theophylline can help mitigate prescribing and medication errors, contributing to optimizing health outcomes for patients with respiratory disorders. Hence, we conducted this study to assess physicians' knowledge about theophylline and its use in Jordan and to identify the variables associated with a higher level of knowledge about theophylline.

MATERIALS AND METHODS

Study design and setting

A survey-based, cross-sectional study of Jordanian physicians was conducted using an online questionnaire between January and April 2023. Licensed physicians practicing medicine in Jordan, from all healthcare sectors and regardless of their specialties, were eligible to participate in the study. No restrictions were applied to the demographic characteristics of the invited physicians, resulting in an overall response rate of approximately 90%.

Eligible physicians were provided with a brief description of the study, and they were informed that their participation was voluntary, with assurances that their responses would be anonymized and kept confidential. Consent to participate was obtained from physicians before they answered the survey questions. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines for reporting cross-sectional studies were used to develop and report this study (23).

Survey development

The survey questions were prepared by the research team based on a review of pertinent literature and international guidelines regarding theophylline use, and they were validated (26-24 ,1). The survey questions were distributed to five clinical pharmacists and five physicians. They were asked to judge the scale items' validity and appropriateness. Their suggestions were taken into consideration. The criterion for accepting an item was the specialist's agreement with an average of 80%. A thorough examination of the literature was conducted to make sure that all relevant criteria were taken into account while establishing the content validity. The paper and questionnaire were located using the source databases Science Direct, PubMed/Medline, and Google Scholar. Next, 40 physicians were assessed using the scale. Since their data was gathered for the pilot, it was not included in the final analysis. The survey questions were prepared and distributed in English language since English is the official language of education for physicians in Jordan. The survey was created and distributed using Google Forms. The validation options "Required" and "Limit to one response" were applied to minimize any cases of missing data and to prevent the duplication of responses.

Flow of the survey questions

The survey consisted of two sections, featuring both open-ended and close-ended questions. The first section comprised demographic questions and other relevant characteristics, including years of experience, place of work, institution, and professional degree. The second section included 16 items designed to assess physicians' knowledge of key information about theophylline, such as indications, mechanism of action, contraindications, and side effects. The final version of the survey, answered by 385 physicians, contained 22 questions. The internal consistency of the study elements was calculated, yielding a Cronbach's alpha of 0.764.

A score of one was assigned for a correct answer on each knowledge item, while a score of zero was given for an incorrect answer. For questions with more than one correct answer, half of one mark was assigned for each correct response. The total score for correctly answered survey items amounted to 30 marks. Subsequently, the knowledge level was categorized into three groups based on the total score: 0-10.5 was classified as Poor knowledge, 11-20.5 as Intermediate knowledge, and 21-30 as Excellent knowledge.

Sample size calculation

In this study, the online Raosoft sample size calculator was utilized to determine the sample size, with a confidence interval of 95% and a 5% margin of error (27). According to the Jordan Medical Association, the number of registered physicians in Jordan was 40,211 in 2022. Therefore, a minimum sample size of 381 was considered representative of physicians in Jordan.

Data analysis

The Statistical Package for Social Sciences (SPSS, version 25.0) software was employed for data analysis. Descriptive statistics, including frequencies and percentages, were performed for all sociodemographic information and knowledge items. Multiple linear regression analysis was conducted to assess the association between sociodemographic characteristics and knowledge scores, with a 95% confidence interval. The total score for each knowledge item was considered the outcome variable. A P-value < 0.05 was considered statistically significant.

Ethical Approval

This study received approval from the Institutional Review Board (IRB) committee of Al-Balqa Applied University in Jordan.

RESULTS

Demographic characteristics of the participants

The demographic characteristics of the 385 physicians who participated in this study are presented in Table 1. The largest share of the participants (46.8%) were less than 30 years old. Males were the predominant participants compared to females (59.2%). Regarding the job title, 160 (41.6%) were residents. Nearly half of the participants, 186 (48.3%), were working in the Ministry of Health. Most participants obtained their medical academic degrees from Jordan (71.9%). About one-third of participants (63%) had experience in the medical field ranging from 2 to 5 years.

Table 1: Demographics characteristics of the participants (n= 385)

Characteristics	n (%)
Age	
Less than 30 years	180 (46.8)
30-39 years	136 (35.3)
40-49 years	43 (11.2)
More than 50 years	26 (6.8)
Gender	
Male	228 (59.2)
Female	157 (40.8)
Region	
North of Jordan	167 (43.4)
Middle of Jordan	84 (21.8)
South of Jordan	134 (34.8)
Place of work	
Ministry of Health	186 (48.3)
Royal Medical Services	115 (29.9)
University Hospitals	38 (9.9)
Private sector	46 (11.9)
Job title	
General Practitioner	96 (24.9)
Resident	160 (41.6)
Specialist	66 (17.1)
Consultant	63 (16.4)
Years of experience	
Less than 2 years	55 (14.3)
2-5 years	137 (35.6)
6-10 years	102 (26.5)
11-15 years	63 (16.4)
More than 15 years	28 (7.3)
Medical academic degrees source	
In Jordan	277 (71.9)
Outside of Jordan	71 (18.4)
Both	37 (9.6)

Physicians' knowledge of theophylline

Table 2 lists participants' knowledge of indications, mechanisms of action, and other related issues of theophylline. Regarding knowledge about theophylline drug, the majority of participants knew that theophylline is used in clinical practice as a bronchodilator (75.6%) and is a non-selective phosphodiesterase inhibitor (75.3%).

When participants were asked about the indications of theophylline, including asthma, chronic obstructive pulmonary disease (COPD), and infant apnea, about half of them knew at least one indication (50.1%). However, 141 participants (36.6%) chose at least one incorrect indication of theophylline. More than half of participating physicians (67.3%) knew that aminophylline is the ethylenediamine salt of theophylline, while 16% of them did not know the relationship between aminophylline and theophylline.

When participants were asked when theophylline should be used in therapy, less than half of the participants (46.7%) chose one correct answer, while one-third of them (36.6%) answered with incorrect answers. Only 5.7% of participants knew all the listed clinical uses of theophylline. Nearly 39% of participants knew that theophylline can be administered orally and intravenously.

Table 2: Physicians' general knowledge of theophylline (n= 385)

Questions	n (%)
Role in clinical practice	
Bronchodilator (Correct answer).	291 (75.6)
Antibiotics or Mucolytics (Incorrect answer).	45 (11.7)
At least one incorrect answer with a correct answer.	45 (11.7)
Don't know.	4 (1)
The primary mechanism of action	
Nonselective phosphodiesterase inhibitors (Correct answer)	290 (75.3)
Cell wall inhibitors or Blocks potassium currents (Incorrect answer).	38 (9.9)
At least one incorrect answer with a correct answer.	19 (4.9)
Don't know.	38 (9.9)
Indication	
One correct answer.	193 (50.1)
Two correct answers.	109 (28.3)
Three correct answers.	13 (3.4)
Incorrect answer.	9 (2.3)
At least one incorrect answer with a correct answer.	55 (14.3)
Don't know.	6 (1.6)
When theophylline should be used in therapy?	
One correct answer.	180 (46.7)
Two correct answers.	22 (5.7)
Incorrect answer.	141 (36.6)
At least one incorrect answer with a correct answer.	26 (6.8)
Don't know.	16 (4.2)
The relationship between aminophylline and theophylline	
Aminophylline is an ethylenediamine salt of theophylline (correct answer).	259 (67.3)
Incorrect answer.	64 (16.6)
Don't know.	62 (16.1)
Route of administration	
One correct answer.	153 (39.7)
Two correct answers.	149 (38.7)
Incorrect answer.	20 (5.2)
At least one incorrect answer with a correct answer.	55 (14.3)
Don't know.	8 (2.1)

Table 3 shows participants' knowledge of multiple aspects of theophylline safety. The majority of participants (71.2%) knew that close monitoring of theophylline should be done regularly. The largest share of participants (76.1%) did not know that theophylline dose is calculated based on the ideal body weight rather than actual body weight, patient's age, or as a fixed dose. Only 25 participants (6.5%) knew that theophylline dose should be adjusted based on serum concentration. On the other hand, more than two-thirds (74.0%) of participants incorrectly thought that the dose is adjusted based on the presence of health conditions, including renal impairment, hepatic impairment, congestive heart failure, hyperthyroidism, or that the dose does not need any adjustments.

Table 3: Physicians' knowledge of theophylline safety (n=385)

Theophylline dose is calculated based on	
Ideal body weight	67 (17.4)
Incorrect answers	293 (76.1)
Don't know.	25 (6.5)
Need for dose adjustment.	
Based on serum theophylline concentration	25 (6.5)
Incorrect answers	285 (74.0)
At least one incorrect answer with a correct answer.	43 (11.2)
Don't know.	32 (8.3)
Common adverse effects at a therapeutic level	
One correct answer.	238 (61.8)
Two correct answers.	80 (20.7)
Incorrect answer.	13 (3.4)
At least one incorrect answer with a correct answer.	18 (4.7)
Don't know.	36 (9.4)
Theophylline toxicity	
One correct answer.	197 (51.1)
Two correct answers.	78 (20.3)
Three correct answers.	40 (10.4)
Incorrect answer.	10 (2.6)
At least one incorrect answer with a correct answer.	31 (8.1)
Don't know.	29 (7.5)
Which of the following drugs interact with theophylline?	
One correct answer.	196 (50.9)
Two correct answers.	67 (17.4)
Three correct answers.	32 (8.3)
Four correct answers.	8(2.1)
Five correct answers.	5 (1.3)
Not having a drug interaction	26 (6.8)
Don't know.	52 (13.5)
Which of the following types of food interact with theophylline?	
Foods high in caffeine, like coffee, tea, cocoa, and chocolate (correct)	253 (65.7)
Vitamin K-rich food (Incorrect answer).	70 (18.2)
Don't know.	62 (16.1)

Close monitoring should be done.	
Regularly	274 (71.2)
Incorrect answers	93 (24.2)
Don't know.	18 (4.7)
Monitoring Parameters	
One correct answer.	160 (41.6)
Two correct answers.	72 (18.7)
Three correct answers.	87 (22.6)
Four correct answers.	8 (2.1)
Incorrect answer.	5 (1.3)
At least one incorrect answer with a correct answer.	25 (6.5)
Doesn't need any monitoring.	4 (1.0)
Don't know.	24 (6.2)
Theophylline use is not contraindicated during pregnancy.	
Yes	231 (60)
No	108 (28.1)
Don't know.	46 (11.9)
Theophylline use is not contraindicated during breastfeeding.	
Yes	105 (27.3)
No	211 (54.8)
Don't know.	69 (17.9)

Participants were asked to indicate common adverse effects and symptoms of theophylline toxicity. More than 60% of participants were able to identify one of the listed adverse effects of theophylline use, including central nervous system and gastrointestinal effects. However, only about 20% knew both adverse effects. About half of the participants (51.1%) knew only one of theophylline toxicity symptoms, including tachycardia, seizures, and cardiac arrhythmia, while only around 10% of them were able to identify all three toxicity symptoms.

Concerning participants' knowledge about drugs that interact with theophylline, around half of the participants (50.9%) knew one correct interacting drug. However, twenty-five of the participants (6.8%) stated that theophylline does not have drug-drug interactions. Drugs that interact with theophylline include antibiotics, phenytoin, allopurinol, benzodiazepines, and oral contraceptives. Nearly two-thirds of participants (65.7%) knew that foods high in caffeine, like coffee, tea, cocoa, and chocolate, have interactions with theophylline. On the other hand, seventy participants (18.2%) stated that vitamin K-rich food has interactions with theophylline, which is an incorrect answer. Participants were asked about the monitoring parameters of theophylline, which include serum theophylline level, heart rate and ECG, respiratory rate, and electrolyte concentrations. Less than half of the participants were able to identify only one correct monitoring parameter, while only 2.1% knew all four monitoring parameters.

Participants were asked whether theophylline can be administered during pregnancy and breastfeeding. A large proportion of participants (60%) knew that theophylline use is not contraindicated during pregnancy. Nevertheless, a notable proportion (28.1%) incorrectly thought that theophylline cannot be given during pregnancy. Regarding theophylline use in breastfeeding mothers, only 27.3% knew

that theophylline use is not contraindicated during lactation, while more than half of physicians (54.8%) incorrectly believed that lactating mothers should not use theophylline.

Physicians' knowledge description

T The participants' knowledge score is presented in Figure 1. The mean knowledge score for the physicians who took part in this study was 13.06 ± 3.78 out of 30 points. The majority of participants (76.1%) had an overall intermediate knowledge of theophylline.

Association between the knowledge score and the different variables

Multivariable logistic regression analyses were performed to identify variables associated with the knowledge score (Table 4). The multiple linear regression model showed that no statistically significant association was found between the sociodemographic characteristics of the participants and the score of knowledge about theophylline.

Table 4: Association between participants' sociodemographic characteristics and knowledge score

Predictors	Knowledge related to Theophylline[1]	
	Beta	P value
Age	0.060	0.585
Less than 30 years		
30-39 years		
40-49 years		
More than 50 years		
Gender	-0.068	0.207
Male		
Female		
Place of work	-0.081	0.150
Ministry of Health		
Royal Medical Services		
University Hospitals		
Private sector		
Region	0.021	0.692
North of Jordan		
Middle of Jordan		
South of Jordan		
General Practitioner	0.199	0.101
Resident		
Specialist		
Consultant		
Years of experience	-0.146	0.149
Less than 2 years		
2-5 years		
6-10 years		
11-15 years		
More than 15 years		
Academic degree source	0.035	0.539
In Jordan		
Outside of Jordan		
Both		

[1]Multiple linear regression.

DISCUSSION

In this study, the level of knowledge among Jordanian physicians regarding theophylline was assessed. Physicians demonstrated an overall intermediate level of knowledge concerning theophylline's role in therapy, administration, and safety issues. This study is the first of its kind in Jordan and the region, addressing multiple aspects of physicians' knowledge about theophylline and highlighting their role in managing patients using theophylline. The survey encompassed physicians' characteristics that could potentially affect their knowledge level, including the place of work, job title, and years of experience. Additionally, the study covered various general aspects of theophylline, such as its role in clinical practice, mechanism of action, indications, and route of administration. It also explored physicians' knowledge regarding theophylline safety aspects, including monitoring, dose calculation, adjustment, adverse effects, toxicity effects, drug-drug, and drug-food interactions, as well as its use during pregnancy and breastfeeding. Overall, studies on the level of knowledge about theophylline are scarce, and most of them involve pharmacists or a combination of pharmacists and physicians, focusing on specific aspects such as drug-drug interactions or indications (28, 29). In contrast, this study exclusively involved physicians and assessed their knowledge across various areas of theophylline use.

The study revealed that a significant proportion of physicians demonstrated adequate knowledge about theophylline's role in clinical practice and its mechanism of action. Satisfactory knowledge in these aspects is reassuring, as it lays the groundwork for effective and safe prescribing practices. However, physicians exhibited limited knowledge of theophylline indications, its clinical uses in therapy, methods of administration, and dose considerations. These knowledge gaps are concerning, as they could lead to suboptimal medication management, compromised therapeutic outcomes, and potential adverse events.

Other noteworthy findings include poor knowledge about theophylline's adverse effects, toxicity, and the required monitoring parameters. Physicians' lack of knowledge and awareness of the medication's possible side effects, toxicity symptoms, and reactions could lead to medication errors, including prescribing errors, inappropriate dosing, and inadequate monitoring. Insufficient knowledge in this area may result in severe consequences such as seizures, tachycardia, arrhythmias, and even death (10, 30). Although a considerable proportion of physicians (71.2%) recognized the need for close monitoring of theophylline, less than half of them (41.6%) could identify only one monitoring parameter, and a mere 2.1% knew all four monitoring parameters. In contrast, a cross-sectional study conducted in Saudi Arabia on the knowledge of healthcare professionals towards therapeutic drug monitoring showed that most of the involved physicians and pharmacists (95%) knew the indications of therapeutic drug monitoring of theophylline (30).

Our findings regarding physicians' knowledge about drugs interacting with theophylline are concerning. While about half of the physicians (50.9%) demonstrated awareness of one correct interacting drug, noticeable proportions (6.8%) incorrectly believed that theophylline does not have interactions with other drugs, and an additional 13.5% did not know whether theophylline interacts with other drugs. These findings are worrisome since theophylline is known to have many significant interactions with commonly prescribed medications such as antibiotics, allopurinol, and oral contraceptives (18, 31).

Similarly, knowledge about food interactions with theophylline revealed mixed results. A majority of physicians (65.7%) correctly identified that foods high in caffeine, such as coffee, tea, cocoa, and chocolate, can interact with theophylline. Conversely, notable proportions incorrectly thought that theophylline interacts with vitamin K-rich foods (18.2%) or did not know of any interacting foods (16.1%). In contrast to our results,

findings from a previous study conducted in South Africa revealed that only a few healthcare professionals working in hospitals knew specific food interactions with theophylline (29). Particularly, only 19.4% of healthcare professionals were aware that patients on theophylline should avoid consuming large quantities of tea, and 21.6% correctly answered about the need to avoid large amounts of chocolates. The disparity in results between the two studies may be attributed to variations in the healthcare professionals included in each study. In our study, the participants consisted solely of physicians, while the other study encompassed a broader range of healthcare professionals, including physicians, pharmacists, dietitians, and nurses. The diverse composition of participants in the other study could have influenced the level of knowledge observed, as different healthcare professionals may have varying degrees of exposure and experience with theophylline interactions.

While a considerable proportion (60%) knew that theophylline could be used during pregnancy, only a few physicians (27.3%) correctly recognized that lactating mothers could use theophylline. This misconception could lead to discontinuing or avoiding a medication that can be useful in the management of respiratory conditions in breastfeeding women.

The identified gaps in physicians' knowledge about the optimal management of theophylline could be due to several factors. First, theophylline possesses a complex pharmacokinetic profile and a narrow therapeutic window, making its appropriate use more challenging (1, 2). Second, physicians might not encounter theophylline as frequently as other medications, therefore, they might have limited exposure and fewer opportunities to practice and update their knowledge about theophylline (32, 33). Moreover, physicians might prioritize retaining knowledge about new medications and widely used therapies while neglecting established medications like theophylline.

This study did not find any significant association between physicians' characteristics and their knowledge score regarding theophylline. The lack of association could be explained by including a limited range of physicians' characteristics, which might limit the ability to detect significant associations. Hence, a broader range of variables could be needed to identify potential associations with knowledge scores.

The main strength of this study is addressing the gap in the literature on the knowledge of physicians regarding theophylline use and safety in the Jordanian context. The study revealed aspects where physicians have insufficient knowledge of theophylline drug. Such findings may have practical implications for optimizing theophylline use and improving patient care and safety. Another strength is the large and diverse sample size of participants. Recruitment of physicians from different areas in Jordan, and from different specialties and healthcare institutions makes the sample more representative of the population of physicians in Jordan and enhances the generalizability of the study findings.

This study has some limitations that should be considered. Our study utilized an online-based questionnaire, introducing a potential source of selection bias. Participants who have a particular interest or concern about theophylline may be more inclined to take part in the survey, leading to self-selection bias. Hence, results should be interpreted while recognizing the potential impact of self-selection on the study's external validity. Further, information about the specialty of the participating physicians was not collected. Accordingly, we are unable to evaluate whether the level of theophylline knowledge varies among different medical specialties. This could affect the interpretation of the study findings since theophylline prescribing practices and familiarity could differ among physicians from various specialties.

CONCLUSION

In conclusion, intermediate knowledge of theophylline was reported among physicians in this study. Physicians demonstrated unsatisfactory knowledge about theophylline's indications, clinical use, administration,

adverse effects, toxicity, interactions with drugs and foods, monitoring parameters, and use in lactation. These findings highlight the need for educational interventions and training programs to improve physicians' knowledge of theophylline and enhance its effective and safe use in clinical practice.

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Informed consent

Informed consent was obtained electronically before answering the questionnaire from all individual participants included in the study.

Conflict of Interest

The authors declare no conflicts of interest. The authors are solely responsible for the content and writing of this paper.

Financial Disclosure

The authors declared that this study received no financial support.

Abbreviations

AAPCC	Association of Poison Control Centers
COPD	Chronic obstructive pulmonary disease
IRB	Institutional Review Board
PDE	Phosphodiesterase

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معرفة الأطباء باستخدام الثيوفيلين: دراسة مقطعية من الأردن

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ملخص

الهدف: تهدف هذه الدراسة المقطعية إلى تقييم مستوى معرفة الأطباء فيما يتعلق بدواء الثيوفيلين والخصائص الأخرى ذات الصلة في الأردن.

المواد والطريقة: أجريت هذه الدراسة بأثر رجعي بين الأطباء في الأردن. تمت مقابلة الأطباء باستخدام استبيان عبر الإنترنت يتكون من قسمين تضمن القسم الأول التركيبة السكانية والخصائص الأخرى ذات الصلة. أما القسم الثاني فيتضمن أسئلة حول دواء الثيوفيلين.

النتائج: أكمل ما مجموعه 385 مشاركا الاستبيان. عرف غالبية المشاركين أن الثيوفيلين يستخدم في الممارسة السريرية كموسع للقصبات الهوائيه (75.6٪). عرف ما يقرب من 39% من المشاركين أن الثيوفيلين يمكن إعطاؤه عن طريق الفم أو الوريد. النسبة الأكبر من المشاركين (76.1%) لم تعلم أن جرعة الثيوفيلين يتم حسابها على أساس وزن الجسم المثالي. عرف 60% من المشاركين أنه يمكن استخدام الثيوفيلين ليس من موانع اثناء الحمل. من ناحية أخرى، فقط 27.3% يعرفون أن استخدام الثيوفيلين ليس من موانع أثناء الرضاعة الطبيعية. غالبية المشاركين (76.1٪) لديهم معرفة متوسطة شاملة بالثيوفيلين..

الاستنتاجات: لوحظ أن الأطباء لديهم معرفة متوسطة شاملة بالثيوفيلين. كان لدى الأطباء معرفة غير مرضية حول مؤشرات الثيوفيلين، والاستخدام السريري، وطريقة الاعطاء، والآثار الضارة، والجوانب الأخرى ذات الصلة. تسلط هذه النتائج الضوء على الحاجة إلى تدخلات تعليمية وبرامج تدريبية لتحسين معرفة الأطباء بالثيوفيلين وتعزيز استخدامه الفعال والآمن في الممارسة السريرية.

الكلمات الدالة: الأطباء، معرفة، الثيوفيلين، الأردن.

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Studying The Anti Candidal-Activity of Different Herbal Oils Incorporated into Tissue Conditioner: (A Comparative study)

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ABSTRACT

This in vitro study was undertaken with the aim of testing the efficacy of the tissue conditioner mixed with four different commercially available herbal oils (Origanum oil, (Nigella sativa) Black seeds oil, Garlic oil, and Ginger oil) against Candida albicans. Control groups tested with antifungal test discs included fluconazole, flucytosine, and tissue conditioner discs (Acrosoft). The susceptibility test of plant extracts alone was also conducted. The combination groups tested were Acrosoft mixed with ginger oil, Acrosoft mixed with origanum oil, Acrosoft mixed with black seed oil, and Acrosoft with garlic oil. Test discs were completely embedded in the tissue conditioner mixed with plant extracts and gently placed on the agar plates. The plates were incubated at 35 °C for 72 hours. After incubation, inhibiting diameters of various groups were noted. There was complete resistance of Candida albicans to both fluconazole and flucytosine, and there was no inhibition zone observed regarding the susceptibility of ginger oil, origanum oil, black seed oil, and garlic oils. Similarly, there was no inhibition of Candida albicans observed in Acrosoft material. For the combination of tissue conditioner with plant extracts, results showed no inhibition in black seed oil and ginger oil combined with tissue conditioner. In contrast, the combination of garlic oil and origanum oil with tissue conditioner showed a zone of inhibition, and the inhibition diameters ranged from 5-9 mm.

Keywords: Antifungal, Herbal Oil, Tissue Conditioner.

INTRODUCTION

There are several methods for replacing missing natural teeth, including complete removable dentures, implants, and overdenture implants, with complete removable dentures being the most common treatment for edentulous patients. However, the oral cavity's microflora plays a crucial role in maintaining the health of gums and throats in individuals with natural teeth or dentures. Denture-related stomatitis (DRS) is a pathological reaction of the denture-bearing mucosa caused by trauma from ill-fitting dentures. If the yeast Candida is involved, the term denture stomatitis is used with the prefix Candida-associated.[1,2] Candida albicans is a diploid asexual fungus that reproduces via budding in culture and tissues, forming pseudohyphae in the process. It can exist in either yeast (blastospore) or mycelial form (pseudohyphae). Denture wearers with normal mucosa typically have the yeast form, while DRS patients exhibit the mycelial form. Candida albicans is a gram-positive yeast and the primary etiological factor in denture stomatitis.[3]

The insertion of a removable prosthesis in the oral cavity induces significant changes in the oral environment, potentially affecting the integrity of oral tissues.[4] Denture-associated stomatitis, one of the most common clinical manifestations of oral candidiasis, affects 24-60% of otherwise healthy denture wearers. Although Candida albicans is the primary cause, other contributing factors, such as bacterial causes, mechanical irritation, and allergies,

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are also present, making the condition a complex entity.[6, 2]

While denture stomatitis is typically asymptomatic, it can cause symptoms such as burning, bleeding, and an unpleasant taste. [7] Fungal infections, including denture stomatitis, are often challenging to cure compared to bacterial infections due to the slow development of fungal organisms. Treatment for denture stomatitis may focus on the oral mucosa, and various successful antifungal medications, both topical (e.g., Amphotericin B and Nystatin) and systemic (e.g., azoles like fluconazole and ketoconazole), have been used. However, systemic antifungal therapy may face challenges related to taste and frequent dosages, impacting patient compliance.[5]

Amphotericin B and Nystatin are commonly used as topical antifungal drugs, while azoles like fluconazole and ketoconazole serve as systemic antifungal therapy. However, the unpleasant taste and the need for frequent dosages associated with these medications have been linked to poor patient compliance.[3]

Douglas and Walker proposed a method of combining tissue conditioner with antifungal medications, specifically mixing Nystatin with tissue conditioner, finding it to be fungicidal to varying degrees. However, reinfection occurred 10 days after treatment discontinuation, prompting further investigation into different antifungal drugs. Tissue conditioners, known for softening the denture-bearing mucosa affected by ill-fitting dentures, act as a cushion beneath dentures, reducing stress on the denture-bearing mucosa.[5]

Tissue conditioners are resilient compounds frequently used to soften the denture-bearing mucosa caused by ill-fitting dentures. They operate as a cushion beneath the dentures, reducing the stresses on the denture-bearing mucosa.[6,7]

Many advantages of employing tissue conditioners as a therapy strategy include no requirement for patient compliance, simultaneous treatment of damaged denture-bearing tissue and candidal infection, and patient convenience because the denture does not need to be removed for an extended period. [8, 9, 10]

Given the advantages of using antifungal compounds in tissue conditioners, a range of medications has been evaluated utilizing various tissue conditioners. The most often used antifungal agent was Nystatin, followed by chlorhexidine, amphotericin, and azole group antifungals such as fluconazole, ketoconazole, miconazole, and itraconazole.[7]

Various chemical substances, including silver zeolite, silver nanoparticles, and magnesium oxide, have been investigated for their antifungal properties in tissue conditioners. Silver zeolite-incorporated tissue conditioners have been shown to manage denture plaque efficiently while remaining unaffected by human saliva.[11,12] In the case of silver nanoparticles, similar outcomes for controlling denture plaque have been observed.[13]

The contemporary natural health trend has contributed to an increase in interest in commercially accessible naturopathic remedies. Medicinal plant extracts have been employed as alternative treatments for health concerns in developing nations.[14]

The primary benefits of natural medicinal plant extracts as antimicrobial agents are increased safety and stability with no adverse effects, which are lacking in both organic and inorganic antimicrobial agents. As a result, natural and herbal products, such as herbal oils, have been investigated for their antifungal properties in addition to organic and inorganic substances. Tea tree oil has been studied for its antifungal activity both in vitro and in vivo.[14] Tea tree oil-modified tissue conditioners exhibited inhibitory and fungicidal action against Candida albicans and are beneficial in treating denture stomatitis. Furthermore, its efficacy was equivalent to that of organic antifungals such as fluconazole.[15]

MATERIALS AND METHODS

Culture methods

Candida albicans test organisms were cultured on Sabouraud dextrose agar plates prepared with Sabouraud Broth base. Muller Hilton agar and 2 percent glucose with

0.5 µg/ml Methylene blue dye (GMB medium) were prepared and used for the sensitivity test.[16] The fungal strains were obtained from the fungal laboratory of the Medical Microbiology Department.

Antifungal assay (Susceptibility test procedure)

GMB medium containing Muller Hilton agar and 2% glucose with 0.5 µg/ml Methylene blue dye was used to prepare 50 plates.[16]

Although ginger oil, origanum oil, black seed oil, and garlic oil are commercially available, they were synthesized in the laboratory for this investigation. Each plant extract stock solution was prepared at a final concentration of 50 µg/ml. The 25 µg per disc concentration was achieved by applying 20 µl of the prepared stock solutions on 6 mm paper discs and allowing them to air dry at room temperature. Subsequently, they were stored in a refrigerator at 4°C.[17] Adjusting the inoculum density of the test fungus using 0.5 McFarland standard tubes, the suspension was then evenly spread onto GMB medium in three directions using a sterile swab. A swab dipped in a standardized inoculum solution was used to streak evenly over plates. The test agents were applied to the surfaces of infected plates using discs. Inverted plates were incubated at 35°C for 72 hours.[18]

Out of the 18 plates, two served as indicators of pure C. albicans development. Antifungal test discs containing fluconazole (flu) and flucytosine (AFY) were evenly distributed on two more plates using sterile tweezers. Two additional plates were used to assess the susceptibility of the tissue conditioner. ACROSOFT TC1 tissue conditioner was created by mixing powder and liquid in a sterile dish according to the manufacturer's suggested ratio, and sterile discs were thoroughly embedded in the mixture. The discs were carefully placed on the agar plate. The susceptibility of plant extracts alone was examined using four plates after achieving the final concentration of 16 µg/ml for each ginger oil, origanum oil, black seed oil, and garlic oil.[19]

The remaining eight plates were used with Acrosoft mixed with ginger oil, Acrosoft mixed with origanum oil, Acrosoft mixed with black seed oil, and Acrosoft mixed with garlic oil. Test discs were thoroughly embedded in the tissue conditioner mix with plant extract and gently placed on the agar plates. The plates were then incubated at 35 °C for 72 hr.[20]

Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Science (SPSS version 22, Chicago, Illinois, USA). Mean, Standard Deviation (SD), and inferential statistics including Shapiro-Wilk, One-way Analysis of Variance (ANOVA), and Tukey's Honestly Significant Difference (Tukey's HSD) were employed, with a significance level set at p-value < 0.05.

RESULTS:

The in vitro study results revealed that the tissue conditioner Acrosoft alone did not exhibit inhibition of C. albicans growth. Additionally, there was complete resistance of Candida to both fluconazole and flucytosine as antifungal agents (Figure 1). No inhibition zones were observed for ginger oil and garlic oil. In contrast, origanum oil and black seed oil showed a non-significant zone of inhibition of 1mm (Figure 5). Table 1 provides the mean and standard deviation for the readings of each type of oil used in this study.

For the combination of tissue conditioner with a plant extract, there was no inhibition observed in the case of black seed oil combined with tissue conditioner. Ginger oil combined with tissue conditioner showed a non-significant zone of inhibition of 1mm (Figure 5).

The study results indicated that the combination of tissue conditioner with origanum oil (Figure 2,3) showed a significant zone of inhibition ranging from 5-9 mm ($p < 0.05$). Similarly, combining tissue conditioner with garlic oil (Figure 4) resulted in a significant zone of inhibition of 9 mm ($P < 0.005$) (Table 1). The overall results of the zone of inhibition for plant extracts and tissue conditioner are presented in Figure 5 and Table 2.

Table (1): Descriptive and statistical test of (the mean of zone of inhibition of *candida albicans*) among groups of herbal oil mixed with tissue conditioner and tissue conditioner alone that had been tested using One-way Analysis of Variance (ANOVA).

Groups of herbal oil mixed with tissue conditioner that had been tested	Mean of zone of inhibition	F	P value
Garlic oil+ Acrosoft tissue conditioner	8±1.732	143.8523	0.0000 Sig.
Black seed oil+ Acrosoft tissue conditioner	0		
Ginger oil+ Acrosoft tissue conditioner	1±0.354		
Organium oil+ Acrosoft tissue conditioner	7.75±1.693		
Acrosoft tissue conditioner	0		

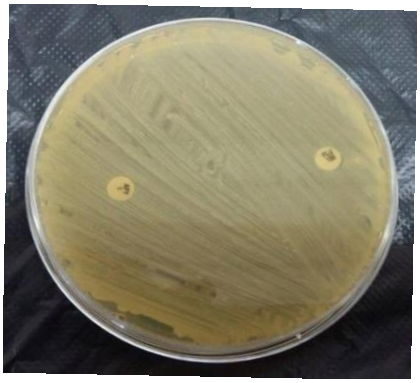

Figure (1): Antifungal susceptibility test for *Candida albicans* against fluconazole and flucytosine

Figure(3): Antifungal susceptibility test for *Candida albicans* against Acrosoft tissue conditioner mixed with Black seed oil (zone of inhibition 5mm)

Figure (2): Antifungal susceptibility test for *Candida albicans* against Acrosoft tissue conditioner mixed with Origanum oil (zone of inhibition 9 mm)

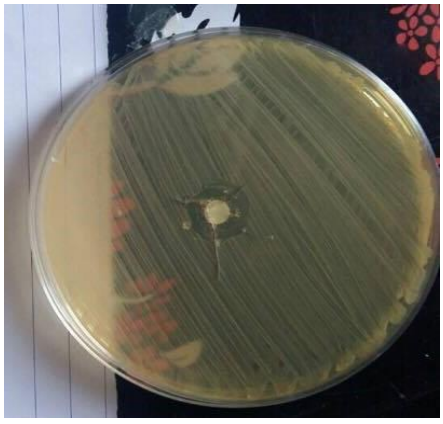

Figure (4)): Antifungal susceptibility test of candida albicance against acrosoft mixed with Garlic oil (zone of inhibition 9mm)

Figure (5): Antifungal susceptibility test for *Candida albicans* against Acrosoft mixed with Ginger oil (zone of inhibition 1mm)

Table (2): Multiple pairwise comparisons of (the mean of zone of inhibition of *candida albicans*) among groups of herbal oil mixed with tissue conditioner and tissue conditioner that had been tested using Tukey's HSD

Groups of herbal oil mixed with tissue conditioner and tissue conditioner that had been tested		**Mean difference**	**P value**
Garlic oil + Acrosoft tissue Conditioner	Black seed oil + Acrosoft tissue conditioner	8.00*	**0.000**
	Ginger oil + Acrosoft tissue conditioner	7.00*	**0.000**
	Organum oil + Acrosoft tissue conditioner	0.25	0.9859
	Acrosoft tissue conditioner	8.00*	**0.000**
Black seed oil + Acrosoft tissue Conditioner	Ginger oil + Acrosoft tissue conditioner	-1.00	0.2629
	Organum oil + Acrosoft tissue conditioner	-7.75*	**0.000**
	Acrosoft tissue conditioner	0	----
Ginger oil+ Acrosoft tissue conditioner	Organum oil + Acrosoft	-6.75*	**0.000**
	Acrosoft tissue conditioner	1	0.2629
Organuim oil +Acrosoft tissue Conditioner	Acrosoft tissue conditioner	7.75*	**0.000**

DISCUSSION

Tissue conditioner is a soft relining substance used therapeutically in the wound healing process. Prolonged use of prostheses has made the site susceptible to microbial buildup. C. albicans, a prominent Candida species isolated from the oral cavity, is closely associated with tissue inflammation known as Candidiasis. Therefore, the incorporation of synthetic or herbal antifungal agents into the tissue conditioner aims to counteract this disadvantage or reduce the risk of acquiring fungal infections.[21]

Medicinal herbs are particularly interesting in developing nations due to their excellent antimicrobial action, safety, and affordability. The recent trend of incorporating extracts of medicinal plants into biomaterials has proven to be a natural alternative with effective antifungal properties.[22,23]

It has been discovered that the methanolic extract of black seeds (Nigella sativa) effectively inhibits fungal development. The antifungal activity of thymoquinone obtained from black seed oil extract was investigated, and the water extract showed substantial action against Candida albicans.[24]

In this study, origanum oil, ginger oil, black seed oil, and garlic oil were added to Acrosoft TC1 tissue conditioner material and evaluated for their fungicidal activity.

Although garlic oil is known for its ability to eliminate a wide variety of pathogens, including fungi, no usage of garlic oil combined with tissue conditioners has been recorded for treating denture stomatitis. The current in vitro investigation indicated that the tissue conditioner Acrosoft did not suppress the development of Candida albicans, which is consistent with the findings of Kulak and Kazazoglu, who observed that the presence of conditioners promoted the adhesion, proliferation, and hyphae production of C. albicans.[25]

The tissue conditioner containing both black seed oil and ginger oil did not prevent yeast growth. This unexpected and unexplainable lack of inhibition can only be traced to a combination of the two medications. On the contrary, the combination of origanum oil and tissue conditioner produced good results, which is consistent with Srivastava's findings that adding origanum oil reduced the adhesion of C. albicans to the surface of the tissue conditioner without affecting its physical qualities.[26] In addition, the inclusion of garlic oil with tissue conditioner demonstrated the predicted efficacy because garlic oil is renowned for its fungicidal properties.

GODIL et al. (2021), using scanning electron microscopy (SEM) studies, also proved the effectiveness of incorporated antifungal agents on the cell morphology of C. albicans at their respective MIC values. This approach permits prolonged drug release in the oral cavity, treating both the injured denture-bearing tissues and the infection biofilms of Candida without compromising their physical properties.[27] This study concurred with the findings of OAamir et al.'s study (2021), which showed that their method permits sustained medication release in the oral cavity without impairing the physical qualities of the denture-bearing tissues and candida biofilms. These studies are important and have enormous medical and therapeutic significance.[27]

In conclusion, the antifungal activity of garlic oil and origanum oil combined with Acrosoft tissue conditioner might be utilized as an alternative treatment for denture stomatitis.[5]

CONCLUSION

The addition of garlic oil and origanum oil to tissue conditioner reduced the growth of C. albicans, suggesting a new intra-oral effective antifungal management for denture stomatitis.

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دراسة الفعالية المضادة للكانديدا للزيوت العشبية المختلفة الداخل فيها مكيف للأنسجة (دراسة مقارنة)

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ملخص

تم إجراء هذه الدراسة في المختبر بهدف اختبار فعالية مكيف الأنسجة الممزوج بأربعة زيوت عشبية مختلفة متوفرة تجاريًا وهي (زيت الزعتر البري، زيت الحبة السوداء، زيت الثوم وزيت الزنجبيل) ضد الكانديدا. كانت مجموعات المراقبة التي تم اختبارها باستخدام أقراص اختبار مضادات الفطريات هي الفلوكونازول، تم إجراء اختبار الحساسية للمستخلصات النباتية وحدها (أقراص فلوسيتوزين ومكيف الأنسجة (أكروسوفت)). كانت المجموعات المركبة التي تم اختبارها عبارة عن اكروسوفت ممزوجًا بزيت الزنجبيل، واكروسوفت ممزوجا بزيت الزعتر البري، وأكروسوفت ممزوجًا بزيت الحبة السوداء، وأكروسوفت مع زيت الثوم. وكانت أقراص الاختبار مدمجة تماما في منعم الأنسجة الممزوج بالمستخلصات النباتية، وتوضع بلطف على أطباق الأجار. تم تحضين الأطباق عند 35 درجة مئوية لمدة 72 ساعة. بعد الحضانة، لوحظ تثبيط أقطار المجموعات المختلفة. وكانت هناك مقاومة كاملة للكانديدا لكل من فلوكونازول وفلوسيتوزين، ولم تكن هناك منطقة تثبيط فيما يتعلق حساسية زيت الزنجبيل، وزيت الزعتر البري ، وزيت الحبة السوداء، وزيوت الثوم. وبالمثل، لم يكن هناك أي تثبيط لوحظ للكانديدا في مادة اكروسوفت . لمزيج منعم الأنسجة مع مستخلص النبات أظهرت النتائج عدم وجود تثبيط في زيت الحبة السوداء وزيت الزنجبيل مع مكيف الأنسجة. على النقيض من ذلك، أظهر مزيج زيت الثوم وزيت الزعتر البري مع مكيف الأنسجة منطقة تثبيط، وتراوحت أقطار التثبيط من 5–9 ملم.

الكلمات الدالة: زيوت عشبية، مكيف انسجة، مضادات الفطريات.

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Discovery of Potential Prolyl-tRNA Synthetase Allosteric Inhibitor Through Virtual Screening and *In Vitro* Assay against *Plasmodium falciparum*

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ABSTRACT

Objectives: This study aimed to identify novel antimalarial compounds based on allosteric inhibitor of prolyl-tRNA synthetase using hierarchical virtual screening.

Materials and Methods: Pharmacophore model was designed initially, based on the structure-activity relationships data between several pyrazole-urea analogues and their IC_{50} enzymatic value. The model obtained was applied to screen ZINC15 database, after which followed by drug-likeness, toxicophore, and PAINS filter. The hit compounds were docked against *P. falciparum* prolyl-tRNA synthetase enzyme, using validated docking method. The resulting docking poses were ranked based on the docking score and re-evaluated based on the pharmacophore criteria. Top five compounds were obtained from this step and then evaluated using molecular dynamics simulation to verify its stability and hydrogen bond dynamics over 50 nanoseconds. MM-PBSA analysis was also performed to estimate their binding free energy. Ultimately, their potential bioactivity as antimalarial candidates have been verified against 3D7 strain.

Results: The results showed that all five compounds obtained from virtual screening possess micromolar potency *in vitro*. Two compounds (ZINC 1029449 and ZINC1029453), yield high antimalarial activity (0.44 and 0.72 μM, respectively)

Conclusions: Overall, the virtual screening approach has successfully produced lead compounds which can be further optimized to be antimalarial agents.

Keywords: Antimalarial, Molecular dynamics, *Plasmodium falciparum*, Prolyl-tRNA synthetase, Virtual screening.

1. INTRODUCTION

Malaria is a global public health concern, particularly in developing countries worldwide[1]. This infectious disease is caused by Plasmodium species, specifically P. falciparum and P. vivax. In 2020, an estimated total of 241 million cases occurred globally, resulting in a 12% mortality rate[2]. Furthermore, several reported case of drug resistances against common antimalarial agent[2] has underlined the necessity to search for alternative therapeutic candidate which is safe and more effective.

High-throughput screening is an integral part of the early drug discovery and development process, allowing the simultaneous assay of multiple compounds at a rate of up to tens of thousands of compounds per week[3]. Due to advancements in computer science and technology, this process can now be simulated in silico, significantly

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reducing the time and resources spent on trial and error in the laboratory while increasing the hit probability for bioactivity screening[4]. This approach, known as virtual screening, has been widely applied with success in generating hits for various biological targets, including the identification of potential compounds with antimalarial activity[5,6]. Virtual screening encompasses various computational tools from different approaches, such as ligand-based methods (pharmacophore, similarity)[7], structure-based method (molecular docking, molecular dynamics)[8–10] or artificial intelligence-based method[11,12]. These tools can be employed subsequently or in parallel to identify the best compounds, which are then tested in vitro. Moreover, this process can be integrated with high-throughput screening to yield more potent lead compounds[4].

Aminoacyl-tRNA synthetases (aaRs) are a family of enzyme which are responsible for esterification of amino acid with cognate tRNA in two-step reaction. Firstly, amino acid will react with ATP to produce amino acid-AMP complex with pyrophosphate anion as side product. Subsequently, hydroxyl group of tRNA attack carbonyl group of amino acid-AMP complex, thus displacing AMP in the complex. The reaction ultimately yields amino acid-tRNA complex, which then delivered to ribosome to take part in protein synthesis. There are 20 aaRS enzymes, which correspond to the total of amino acid in nature[13]. This enzyme has garnered some interest recently, notably as potential druggable target in various infectious diseases such as malaria[14]. To date, 36 aminoacyl-tRNA synthetases are known to reside inside apicoplast, mitochondria, or cytoplasm of *Plasmodium falciparum*, of which five enzymes have been structurally characterized[14]. Prolyl-tRNA synthetase (PfPRS) is one of the examples. Its significance was first known in the 2010s as the main target of febrifugine, halofuginone, and their other derivates activity [15–18]. Crystallographic data shows that febrifugine and its analogues inhibit PfPRS by occupying tRNA and L-proline binding site [16,18].

This dual site binding mechanism is observed not only in *Plasmodium falciparum* but also in human orthologue (HsPRS) [18,19], due to the very high homology between the two. Upon examination, it can be observed that PfPRS shares around 54% similarities with HsPRS. The difference lies in the zinc binding motif, which exists only in HsPRS. A slight deviation can also be found in anticodon binding domain [18]. Nevertheless, it is shown that febrifugin-like compound binds in the same manner on both orthologues, making their selectivity questionable.

Recent study showed novel binding mode of PfPRS via allosteric regulation, which yield higher selectivity against HsPRS. Based on high-throughput screening result, it was found that pyrazole-urea based compound possess selective activity towards PfPRS and promisingly potent scaffold against *Plasmodium falciparum* [20] **(Figure 1)**. This allosteric ligand is in the vicinity of ATP binding site, specifically in the TXE loop. In the process, it displaces the loop from the conservative conformation [18] **(Figure 2).**

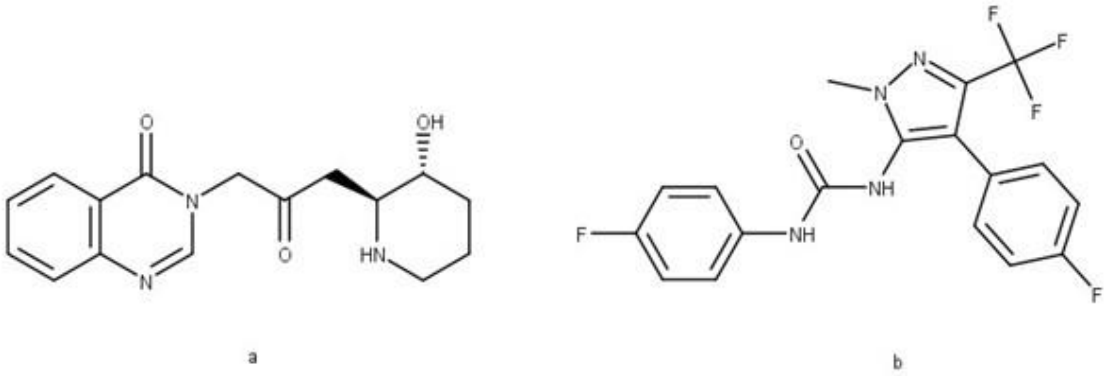


Figure 1. Chemical structure of febrifugine (a) and TCMDC-124506 (b), an orthosteric and allosteric inhibitor of PfPRs enzyme, respectively.

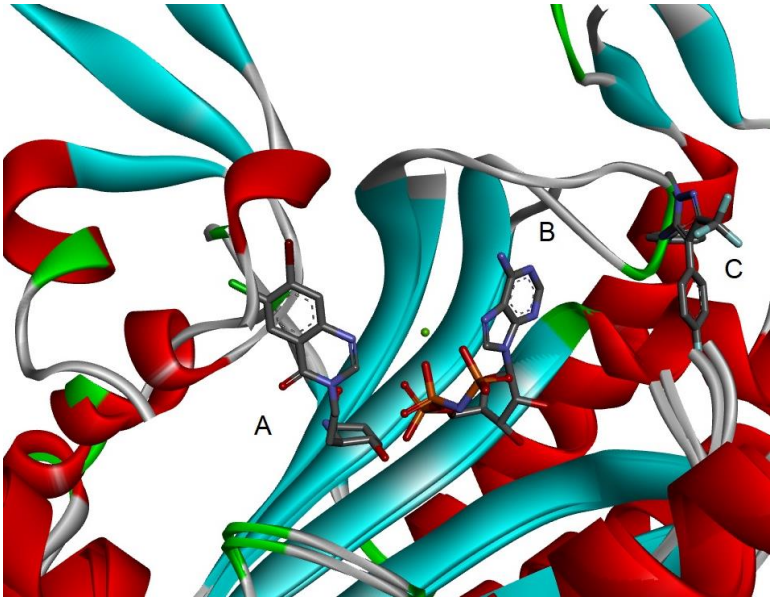


Figure 2. Overlay image of two PfPRS crystal structure containing halofuginone-AMPPNP and TCMDC-124506, respectively. (A=halofuginone; B=AMPPNP, an ATP analogue; C=TCMDC-124506)

In addition, several plant-based compounds have been predicted to possess specific enzymatic activity toward PfPRS using virtual screening and molecular dynamics[21]. In this study, a similar approach was implemented in attempt to identify potential selective PfPRS inhibitor among commercially available compounds in ZINC database[22]. Ultimately, antimalarial activity of the compounds obtained through this process were verified by *in vitro* assay against *Plasmodium falciparum* strain 3D7.

2. METHODS

2.1. Phamacophore Modelling and Screening

Pharmit webserver (https://pharmit.csb.pitt.edu/) was used for virtual screening[23]. Our protocol commenced with structure-based pharmacophore modeling using crystallographic data of PfPRS with allosteric inhibitor **(PDB ID: 4WI1)** [20]. The predetermined pharmacophore query from webserver was then modified according to known information of their structure-activity relationship[20]. Resulting pharmacophore model was then applied to screen 13,190,317 compounds from ZINC purchasable database[22]. This procedure yielded 248 hit molecules, which were proceeded to the next step.

2.2. Drug-likeness, Toxicophore, and PAINS Filtering

The obtained compounds from previous step were filtered based on Lipinski rule of five[24] to assess their drug-likeness. In addition, possessing unwanted moieties, such as toxicophores and PAINS, were targeted for exclusion. This step was performed using FAF-Drugs 4 webserver (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::FAF-Drugs4)[25] The aim was to ensure that the obtained compounds are drug-like, free of toxic functional groups, and potentially not possessing promiscuous bioactivity. Criteria for defining toxic and unwanted moieties are explained in [25], while for definition of PAINS substructure are according to [26]. Notably, no compounds were found to violate all the rules. Consequently, 248 molecules proceeded to the next step.

2.3. Molecular Docking

Molecular docking step was performed using the filtered compounds from previous step and the same protein from pharmacophore modelling process (PDB ID: 4WI1) [20]. Prior to performing molecular docking, ligand and protein preparation was performed to ensure both of protein ligand represent the real condition as accurate as possible. This preparatory step includes adding hydrogen atom and partial charges of Amberff14 [27] and Gasteiger [28] for protein and ligand, respectively. The whole process of protein preparation was done in Chimera 1.14 [29] while ACC2 was used to compute partial charge of all ligands [30].

The following process was validation step. This was done to ensure the reliability of the docking method. Our approach was to evaluate the best combination of docking score and placement algorithm available in Molegro 7 Trial Version (http://molexus.io/molegro-virtual-docker/) which was used as docking software. There were two procedures of validation took place in this step. Firstly, the native ligand (TCMDC-124506) of the enzyme was removed and subsequently re-docked into the enzyme (*i.e.* self-docking/re-docking). The resulting docking pose was then superimposed to the original conformation and calculated their RMSD value, which ideally should not be over 2.0 Å[31]. Afterwards, molecular docking was performed against data set of ligands which contained both known active and inactive compounds from literature [20]. The lowest docking score obtained from each ligand was sorted ascendingly and the overall ranking was evaluated based on its area under curve (AUC) value of ROC curve [32], BEDROC [33], and standardized total gain score [34]. This calculation was done in Screening Explorer webserver [35]. The docking process was conducted in 15 Å-radius spherical region centered on native ligand.

Afterwards, the selected best method was applied to dock 248 hit molecules. The result was ranked ascendingly and evaluated subsequently according to the SAR report [20]. Five of the compounds who met the criteria, in addition to possess low docking score were selected to be processed.

2.4. Molecular Dynamics and MM-PBSA Calculation

The selected compounds from molecular docking step and the native ligand (TCMDC-12506) were then simulated using Gromacs 2016.3 simulation pack [36]. Similar forcefield and partial charge (Amberff14 [27] and Gasteiger [28]) was applied in the preparatory stage, before the docked complexes were subjected to 50 ns simulation in water and counterions (Na^+ & Cl^-). TIP3P rigid water model [37] was used in this study for its computational speed and reasonable accuracy in protein-ligand simulation [38].

Long-range electrostatic force was determined by Particle Mesh Ewald [39]. Velocity rescaling thermostat [40] and Parrinello-Rahman barostat [41] were used during NVT and NPT equilibration for 500 ps, respectively. In these processes, system temperature was adjusted to 310 K, while maintaining the pressure at 1 bar. Molecular dynamics production run was performed in a 2 fs timestep for 50 ns. The stability of the system was verified by analysis of the energy, temperature, pressure, and root-mean-square deviation (RMSD).

Afterwards, MM-PBSA calculation was performed using the G_MMPBSA package integrated in the Gromacs 2016.3 software[42]. Polar desolvation energy was calculated with the Poisson-Boltzmann equation with a grid size of 0.5 Å. The dielectric constant of the solvent was set to 80, which represents water as the solvent. Non-polar contribution was determined by calculation of the solvent-accessible surface area with the solvent radii of 1.4 Å. The binding free energy of the complex was determined based on 50 snapshots taken from the beginning to the end of the molecular dynamic simulation trajectories of the complexes.

2.5. Antimalarial Bioassay

The compounds obtained from virtual screening process were purchased from MolPort (Riga, Latvia) to be tested for their antimalarial potency. Antimalarial assay was conducted against *Plasmodium falciparum* strain 3D7. Parasites were bred in human erythrocyte using Trager-Jensen method with slight modification [43,44]. Each assay compounds were dissolved in DMSO to make 10 ppm solution. This stock solution was diluted into four other concentrations (1, 0.1, 0.01, and 0.001 ppm). 500 µL aliquot of solution was mixed with the equal amount of parasite culture in a 96 well plate, then incubated for 48 h at 37°C. This process was conducted for all five different concentrations. Chloroquine diphosphate was used as positive control. In addition, negative control was also measured using parasite culture only. Plasmodium growth was evaluated in microscope using thin blood smears preparation with Giemsa stain. Inhibition percentage can be calculated using the following equation:

$$\%\ Inhibition = \frac{(100\% - A)}{B}\ x\ 100\%$$ [44].

Where A and B refers to the growth percentage of compounds and negative control, respectively. Ultimately, IC_{50} values were calculated by transforming the concentration-response curve using the Probit Transformed Responses regression model. The values were expressed as a mean value with standard deviation.

3. RESULTS AND DISCUSSION

Virtual screening is currently becoming one of the most powerful tools to aid drug discovery process in cost and time-efficient manner. The method combines various drug design tools into a systematic workflow which act as a filter for the chemicals in library. This will increase the probability of finding hit and eliminate likely inactive compound[4]. There are several types of virtual screening algorithm based on their level of integration, one of which is hierarchical or classical virtual screening as implemented in this study[45]. Here we applied pharmacophore modelling, molecular docking, and molecular dynamics in a sequential order to obtain the most potentially active compounds against PfPRS enzyme.

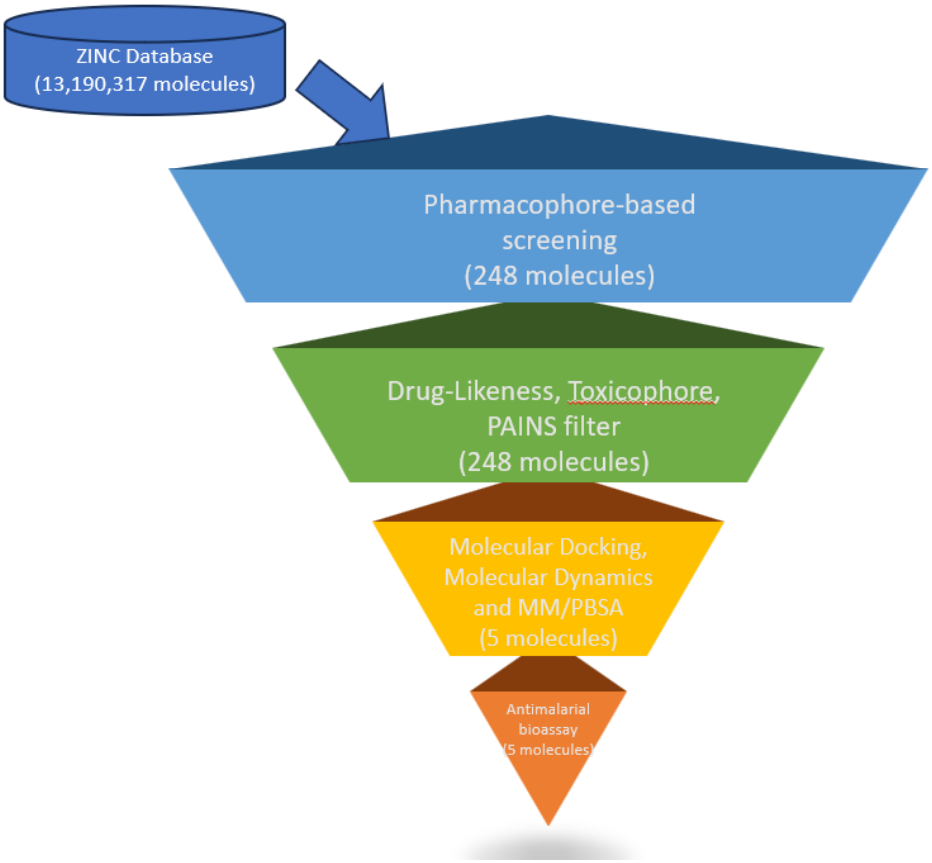


Figure 3. Workflow of virtual screening used in this study

In the beginning, pharmacophore model was built based on structure-activity relationships of pyrazole-urea analogues against PfPRS enzyme. The model was built using TCMDC-124506 as a template [20]. It consists of two aromatic ring queries on pyrazole and phenyl moiety attached to it, one hydrogen bond acceptor and two hydrogen bond donors on urea moiety, and two hydrophobic queries on N-substituent position of pyrazole and ring moiety attached to urea group. The purpose of implementing hydrophobic query instead of aromatic ring for the latter is due to the fact that glibenclamide, which contains a hydrophobic moiety, also known to possess activity against PfPRS, comparable to the TCMDC-124506 [20] (Figure 4). The resulting pharmacophore model was then used to screen ZINC database. This process has yielded 248 molecules. All of these compounds were also passed FAF-Drugs 4 filter of toxicophore and PAINS substructure [25], ensuring the absence of potentially toxic and/or frequent-hitter compound [26].

Subsequently, molecular docking process was performed towards those compounds. Validation of this process was carried out to select the best algorithms available in Molegro 7. This docking software has three placement scorings and four docking scores. Initially, we evaluated those 12 combinations according to their RMSD value. The result showed that all but one algorithm produced docking pose with acceptable RMSD value (Figure 5).

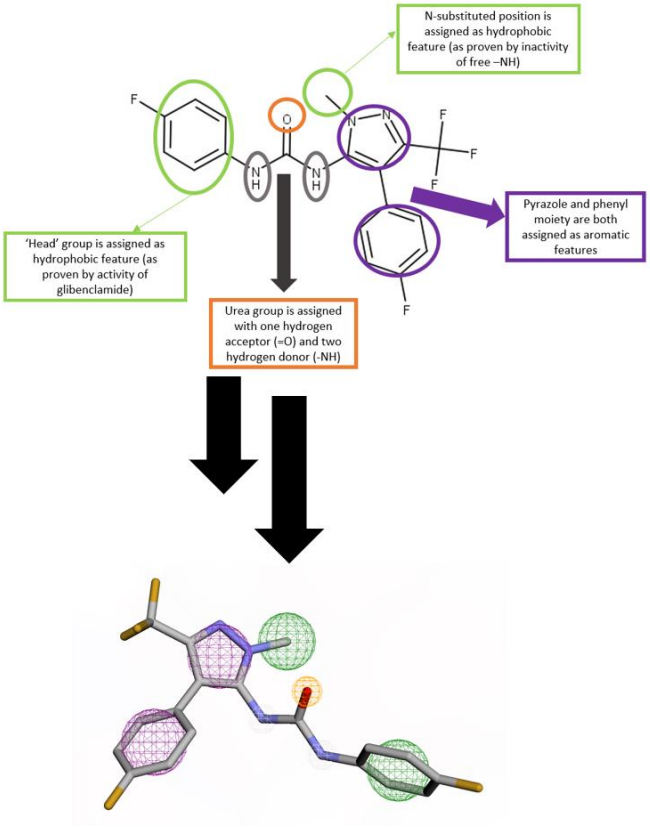
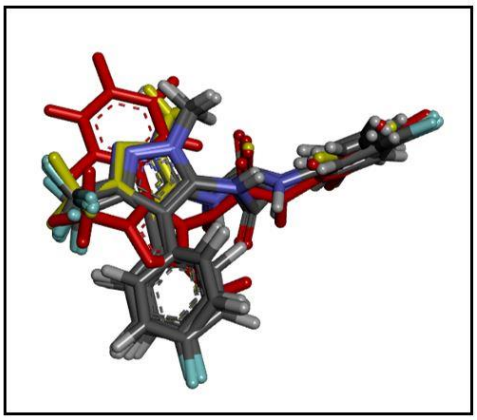


Figure 4. Pharmacophore queries of PfPRS inhibitor according to [18] (top) and its three-dimensional visualization using Pharmit webserver (bottom)



Scoring/Placement	MolDock Optimizer	MolDock SE	Iterated Simplex
MolDock Score	0.18	1.02	0.19
MolDock Score (Grid)	0.47	0.97	0.21
PLANTS Score	0.18	0.19	0.24
PLANTS Score (Grid)	3.92	0.16	0.21

RMSD Threshold ≤ 2.0 Å (RMSD value in red exceeds the threshold)

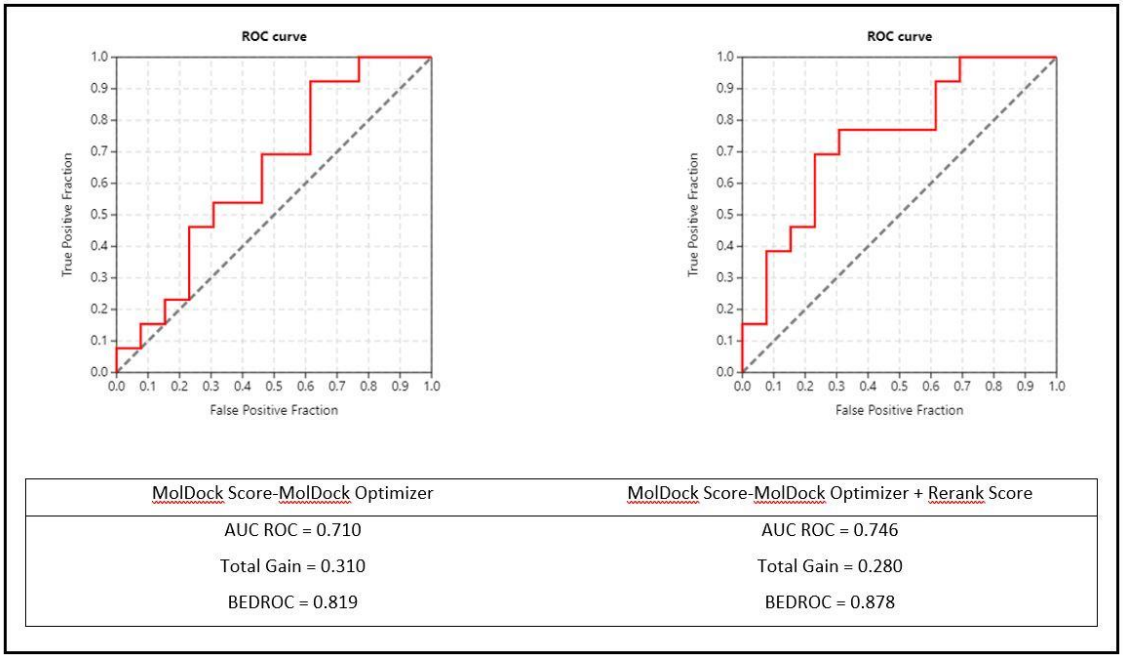
Figure 5. RMSD values calculated for 12 algorithms against PfPRS enzyme (PDB ID: 4WI1) and the superimposed ligand conformations of all the algorithm (yellow: native ligand; red: re-docking result with RMSD > 2.0 Å

The next step was to evaluate whether a method could discriminate between active and inactive compounds based on docking score-based ranking. In this context, we conducted molecular docking against analogs of pyrazole-urea whose enzymatic activity had been determined previously[20]. An alternative approach involved using putative inactive compounds, i.e., decoy compounds, as substitutes due to insufficient data on inactive compounds[46]. The evaluation was carried out based on the area under the curve values of ROC and BEDROC, as well as the Total Gain value. The ROC curve has been widely used in numerous studies as a validation tool in virtual screening campaigns[32,47,48]. This metric ranges from 0 to 1, representing the complete inability and perfect capability of a method to separate active and inactive compounds, respectively[49]. BEDROC is a modification of the ROC curve that applies Boltzmann distribution to enhance its ability to discriminate early hits in virtual screening [31]. Meanwhile, Total Gain is a statistical tool used to quantify the score of the virtual screening process in explaining compound bioactivity. This parameter is akin to the determination coefficient, where the value ranges from 0 to 1, representing the explanatory power of the virtual screening method[34,50]. From this validation step, it was found that only one algorithm (MolDock Score-MolDock Optimizer) works best to enrich active molecule and in accord with all validation metrics **(Table 1)**. MolDock Score is a docking score based on piecewise linear potential (E_{PLP}) with additional terms namely hydrogen bonds direction [51]. MolDock Optimizer is a placement algorithm based on differential evolution algorithm. This method is identical to genetic algorithm, albeit the result is more guided by addition of weighted difference of previous calculation [51].

Table 1. AUC ROC, Total Gain, and BEDROC values calculated for 11 algorithms against PfPRS enzyme (PDB ID: 4WI1)

Algorithms	AUC ROC	Total Gain	BEDROC
MolDock Score- MolDock Optimizer	**0.710**	**0.310**	**0.819**
MolDock Score- MolDock SE	0.562	0.090	0.205
MolDock Score- Iterated Simplex	0.432	0.067	0.280
MolDock Score (Grid)- MolDock Optimizer	0.615	0.157	0.161
MolDock Score (Grid)- MolDock SE	0.568	0.188	0.136
MolDock Score (Grid)- Iterated Simplex	0.574	0.081	0.720
PLANTS Score- MolDock Optimizer	0.651	0.171	0.343
PLANTS Score- MolDock SE	0.568	0.170	0.150
PLANTS Score- Iterated Simplex	0.408	0.148	0.201
PLANTS Score (Grid)- MolDock SE	0.645	0.233	0.312
PLANTS Score (Grid)- Iterated Simplex	0.503	0.074	0.618
Acceptable Threshold	>0.50	>0.25	>0.50

The virtual screening output can be further enhanced by applying a consensus scoring approach[52]. In this context, we incorporated the Rerank Score in addition to the MolDock Score to increase the discriminative power between active and inactive compounds. This method falls under the category of weighted sum ranking[52], where the existing docking score is modified by the Lennard-Jones 12-6 potential to better depict steric factors[51]. The results showed a significant improvement based on both AUC-ROC and BEDROC values (Figure 6), signifying better early recognition of active compounds[32,33].

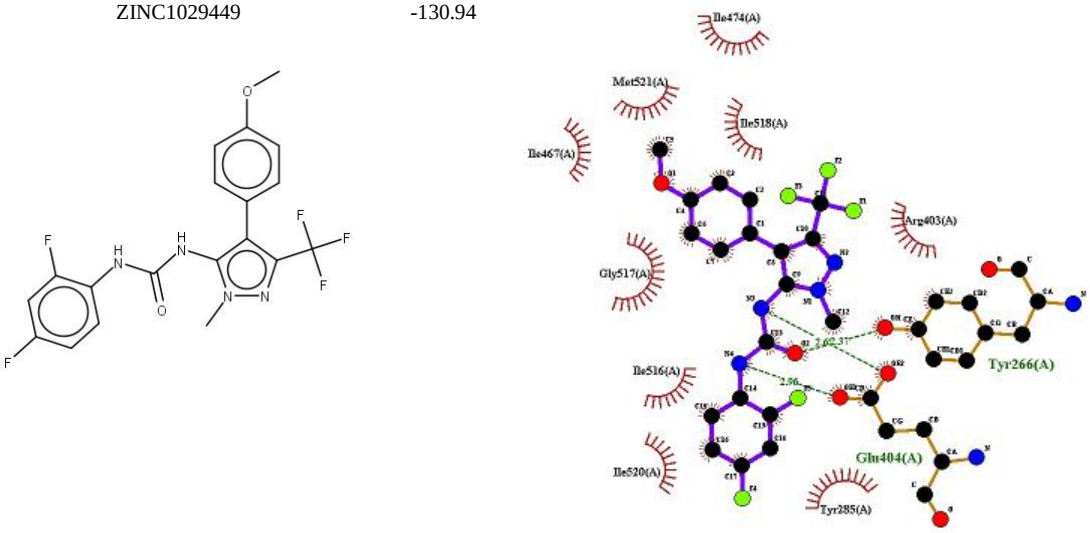
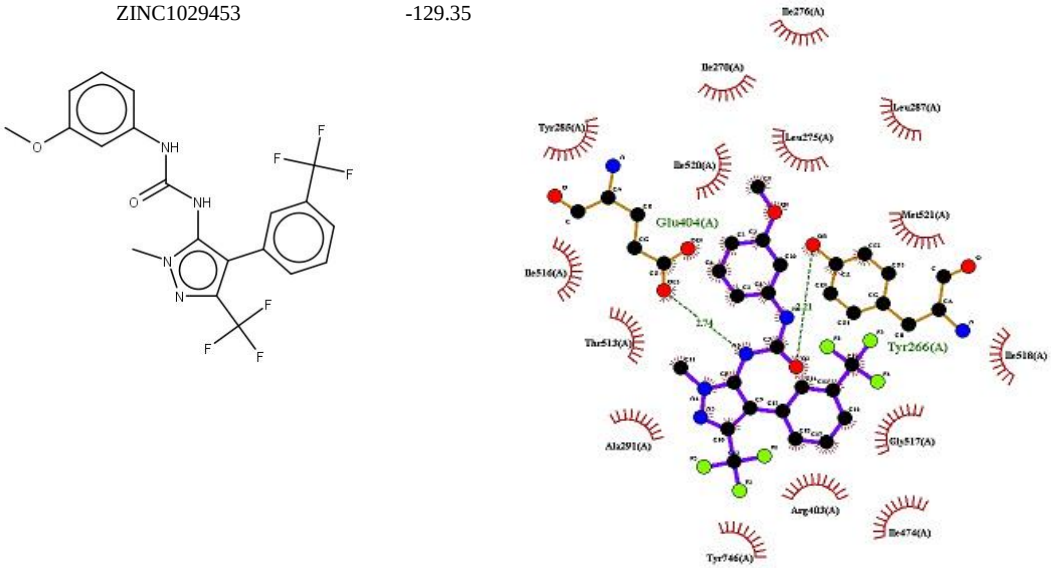


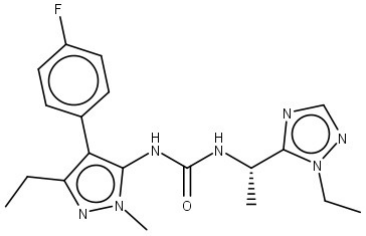
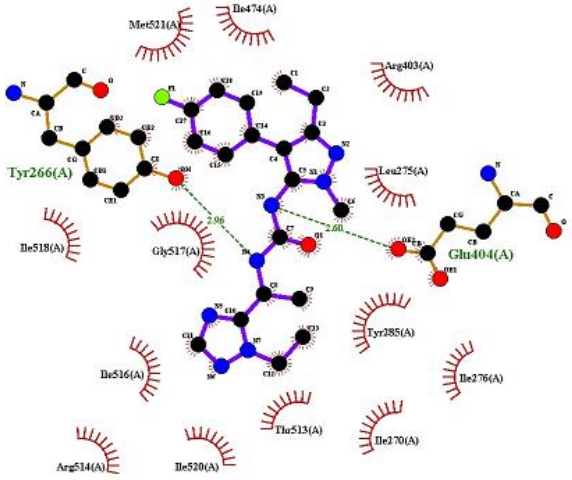
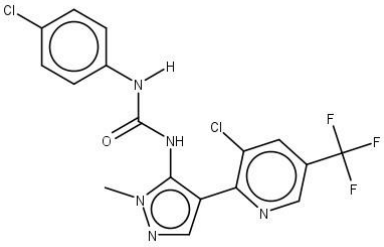
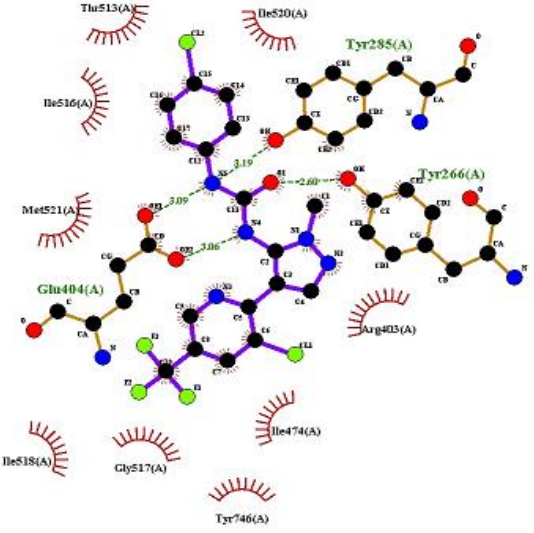
MolDock Score-MolDock Optimizer	MolDock Score-MolDock Optimizer + Rerank Score
AUC ROC = 0.710	AUC ROC = 0.746
Total Gain = 0.310	Total Gain = 0.280
BEDROC = 0.819	BEDROC = 0.878

Figure 6. Validation result of MolDock Score-MolDock Optimizer (left) and MolDock Score-MolDock Optimizer with the implementation of Rerank Score (right)

This method was then applied to dock 248 compounds obtained from pharmacophore screening. However, post-docking evaluation revealed that several high-ranked compounds possess a free NH pyrazole moiety. We decided not to select these compounds since they contradict the pharmacophore model, which specifies a N-substituted pyrazole ring. Therefore, a manual inspection was performed to choose five compounds in an ascending manner that conform to the pharmacophore model. It can be observed that most of the obtained compounds possess a pyrazole-urea moiety, with only one compound containing an isoxazole scaffold in place of pyrazole (Table 2). Overall, the compound bearing the pyrazole-urea group ranked better than the isoxazole-urea based on their MolDock Score. These five docked compounds and TCMDC-124506 were then subjected to a 50 ns molecular dynamics simulation and MM-PBSA analysis to evaluate their conformational dynamics, structural stability, and free binding energy with the solvation model. Several parameters were evaluated post the molecular dynamics process, such as the RMSD value of the protein, RMSF plot of amino acids, and hydrogen bond occupancy of all protein complexes.

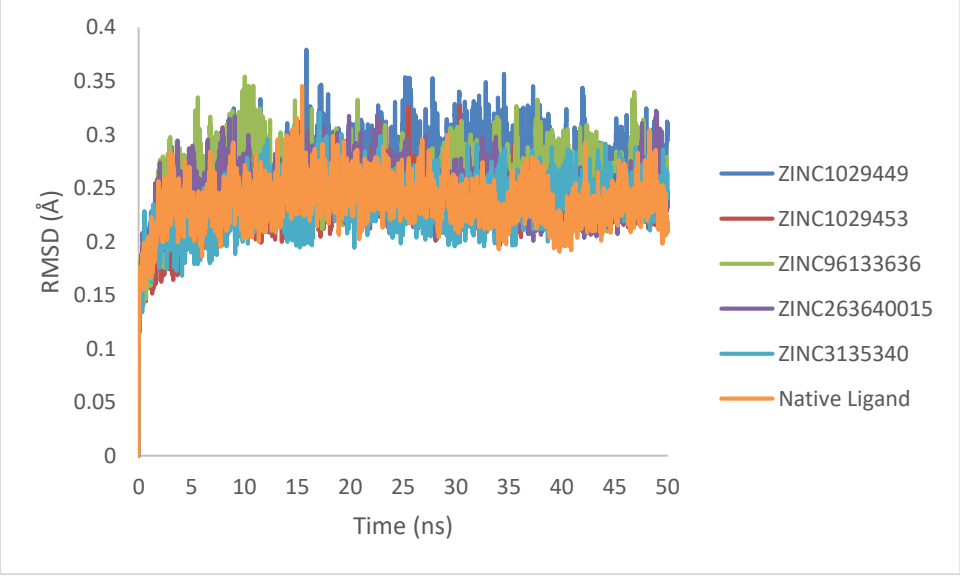
Table 2. Docking score and ligand interaction result of selected compounds

Compounds	MolDock Score +Rerank Score	Ligand Interaction*
ZINC1029449	-130.94	
ZINC1029453	-129.35	

Compounds	MolDock Score +Rerank Score	Ligand Interaction*
ZINC96133636 	-126.72	
ZINC263640015 	-121.68	

Compounds	MolDock Score +Rerank Score	Ligand Interaction*
ZINC3135340	-120.17	

* Ligand interaction was evaluated using LigPlot+ 2.2.4 [45] (Laskowski and Swindells, 2011).



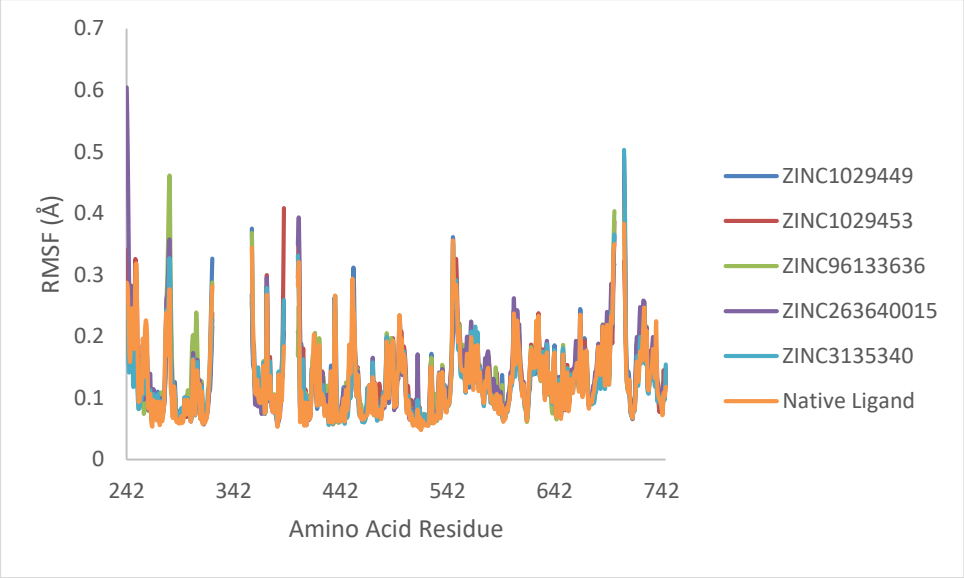


Figure 7. RMSD (above) and RMSF (below) plot of protein during 50 ns simulation

The RMSD values of the protein observed during the 10-50 ns simulation indicated the stability of all complexes. The RMSD fluctuation plot over simulation time suggested that all protein systems had reached convergence by the end of the molecular dynamics process (Figure 6). Subsequently, RMSF plots were evaluated to observe protein residue flexibility during molecular dynamics simulation. The results showed high peaks, notably in the β-hairpin structure (residue 279-283) in the catalytic domain region (CD) and the loop-α-helix structure (residue 547-554) in the anti-codon binding domain (ABD) (Figure 7).

Hydrogen bond occupancy percentage was calculated to illustrate the dynamic process of hydrogen bond interaction during the 50 ns simulation. The calculation was performed using HBonds 1.2, an integrated plugin from VMD[53]. The results, calculated as a percentage, indicate the frequency of hydrogen bond formation during molecular dynamics simulation. It was observed that, similar to the amino acid interaction in the molecular docking process, interactions with Glu404 and Tyr266 were consistently found in almost all ligand-protein complexes. The occupancy values sometimes exceeded 100%, as seen in Glu404 interaction with ZINC102949 (116.87%). This type of interaction was also observed in the native ligand (TCMDC-124506), emerging as the only distinctive hydrogen bond interaction during the 50 ns simulation, underscoring its significance in ligand-protein interaction.

Several novel hydrogen bond interactions were also elucidated during the simulation process, such as in ZINC3135340, which formed a hydrogen bond with Thr513 and Phe405. On the other hand, it appears that two of the ligands (ZINC96133636 and ZINC263640015) showed lower values of hydrogen bond occupancy compared to the rest of the compounds, indicating a different type of ligand-amino acid interaction could take place (Table 3). Observation of the final MD snapshots also indicated several changes in ligand interaction, namely new hydrogen bond formation between ZINC3135340 and Phe405 or the absence of hydrogen bond interaction in ZINC 96133636 (Table 3). This result

generally aligns with the hydrogen bond occupancy values during the 50 ns (Table 2), where hydrogen bonds with high percentage values will be observed more frequently than the lesser ones.

Afterward, we also calculated the binding free energy of all ligands using the MM-PBSA approach. It is one of the commonly used methods to estimate ligand free energy values, aside from MM-GBSA, LIE, and alchemical binding[54–56]. This approach is an amalgamation of energy calculation based on molecular mechanics and implicit solvent-based free energy calculation, as explained in the following equation.

$$\Delta G_{binding} = [(\Delta E_{bonded} + \Delta E_{electrostatic} + \Delta E_{van\ der\ Waals}) + (\Delta G_{polar\ solvation} + \Delta G_{surface\ area})] - T\Delta S$$

Table 3. Hydrogen bond occupancy analysis post-molecular dynamics simulation

Compounds	Hydrogen Bond Donor Occupancy*	Hydrogen Bond Acceptor Occupancy*
ZINC1029449	Tyr266 (s) (46.08%) Phe405 (m) (0.16%) Arg403 (s) (0.04%)	Glu404 (s) (116.87%) Arg403 (m) (0.02%)
ZINC1029453	Tyr266 (s) (57.30%)	Glu404 (s) (0.26%)
ZINC96133636	Gly283 (m) (3.22%) Tyr285 (s) (2.38%) Arg403 (s) (0.32%) Thr513 (s) (0.26%) Phe405 (m) (0.02%)	Thr513 (m) (0.02%) Tyr285 (s) (0.02%)
ZINC263640015	Tyr266 (s) (13.49%) Tyr278 (s) (7.84%) Arg514 (s) (0.02%)	Glu404 (s) (3.12%)
ZINC3135340	Thr513 (s) (29.93%) Phe405 (m) (23.57%) Tyr266 (s) (5.76%) Tyr285 (s) (0.16%) Leu406 (m) (0.06%)	Glu404 (s) (52.64%) Tyr285 (s) (5.42%) Arg403 (m) (1.84%)
TCMDC-124506	Tyr266 (s) (58.76%)	Glu404 (s) (63.5%)

* s = side-chain hydrogen bond; m = main hydrogen bond

The first three variables in the equation refer to molecular mechanic energy (MM), which consists of bonded and non-bonded interactions (electrostatic and van der Waals). Meanwhile, the free energy terms are made up of the total polar and non-polar contributions. In the g_mmpbsa module, these are obtained from the Poisson-Boltzmann equation (PB) and solvent-accessible surface area (SA) value, respectively[42]. MM-PBSA approach is arguably time efficient[54] and has been implemented numerous times in virtual screening approaches to improve the reliability of protein-ligand interaction evaluation[55]. Based on the MM-PBSA calculation for 50 ns, it is observed that the compound ZINC1029449 from molecular docking possesses better binding free energy

than the rest of the compounds, including the native ligand (Table 4). We argue that hydrogen bond interaction with Glu404 plays an important role in yielding better binding free energy, followed by the Tyr266 hydrogen bond.

Ultimately, the compounds were tested for their antimalarial potency in vitro against Plasmodium falciparum chloroquine-sensitive strain (3D7). This parasite strain was chosen as it is known to express PfPRS enzyme[16,57]. According to the previous study[20], it can be expected that pyrazole-urea analogs yield antimalarial activity. The results we obtained indicate that all our assayed compounds possess micromolar inhibitory activity (Figure 8), with the top two compounds from in silico evaluation (ZINC 1029449 and ZINC1029453) being the most potent inhibitors with IC50 values of 0.44 and 0.72 µM, respectively.

Table 4. Binding free energy of protein-ligand interaction calculated by MM-PBSA

Compounds	$\Delta G_{binding}$	$\Delta E_{van\ der\ Waals}$	$\Delta E_{electrostatic}$	$\Delta G_{polar\ solvation}$	SASA
ZINC1029449	-137.146 ± 14.941	-212.072 ± 12.999	-84.083 ± 9.288	180.192 ± 16.496	-21.183 ± 0.713
ZINC1029453	-116.017 ± 15.494	-199.007 ± 15.776	-31.093 ± 13.597	135.192 ± 22.750	-21.108 ± 1.036
ZINC96133636	-102.922 ± 15.112	-209.159 ± 12.609	-5.622 ± 11.764	131.976 ± 16.185	-20.117 ± 0.868
ZINC263640015	-109.242 ± 17.206	-175.256 ± 15.315	-23.208 ± 16.997	107.706 ± 26.512	-18.483 ± 1.180
ZINC3135340	-109.879 ± 16.684	-180.185 ± 15.465	-93.073 ± 23.940	181.694 ± 24.243	-18.315 ± 1.153
TCMDC-124506	-117.262 ± 13.454	-187.746 ± 12.215	-79.158 ± 11.680	169.302 ± 17.442	-19.660 ± 0.813

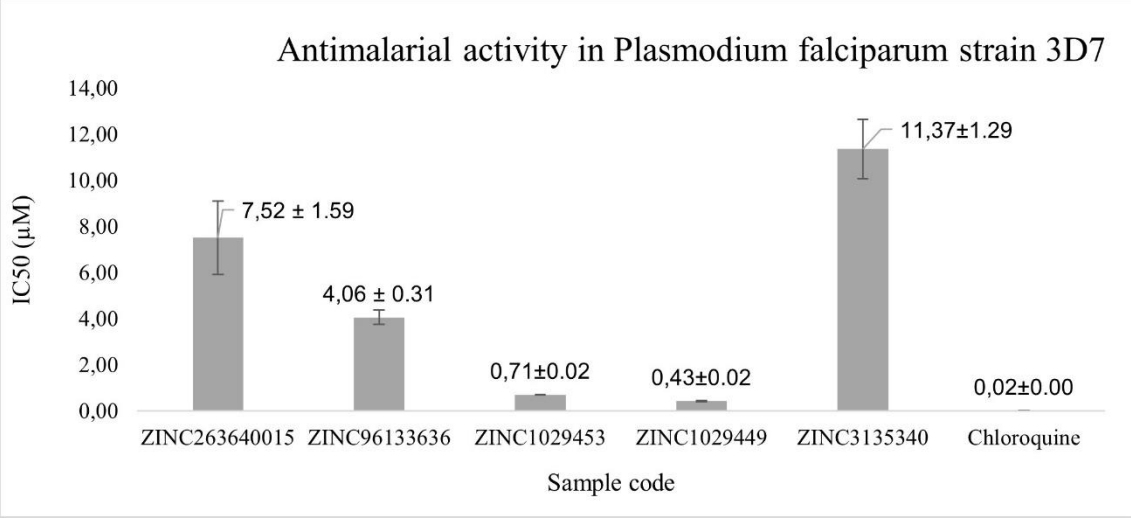


Figure 8. Antimalarial activity of five tested compounds against *Plasmodium falciparum* 3D7

All compounds bearing the pyrazole-urea scaffold perform better than the isoxazole-urea one. It is also worth noting that the in vitro assay result was generally in line with the docking score value, and the top two ranked compounds in terms of binding free energy are identical to the antimalarial assay. Compounds ZINC 1029449 and ZINC1029453 have a similar scaffold to TCMDC-124506 and its analogs, which have been tested for their antimalarial potency against both PfPRS enzyme and the 3D7 strain[20]. On the other hand, it is also found that several modifications of the 'head' and phenyl 'tail' group of the pyrazole-urea analogue slightly lower the antimalarial bioactivity. We also found that the substitution of the pyrazole moiety with the isoxazole ring has significantly reduced its potency, as shown by compound ZINC3135340 (Figure 9).

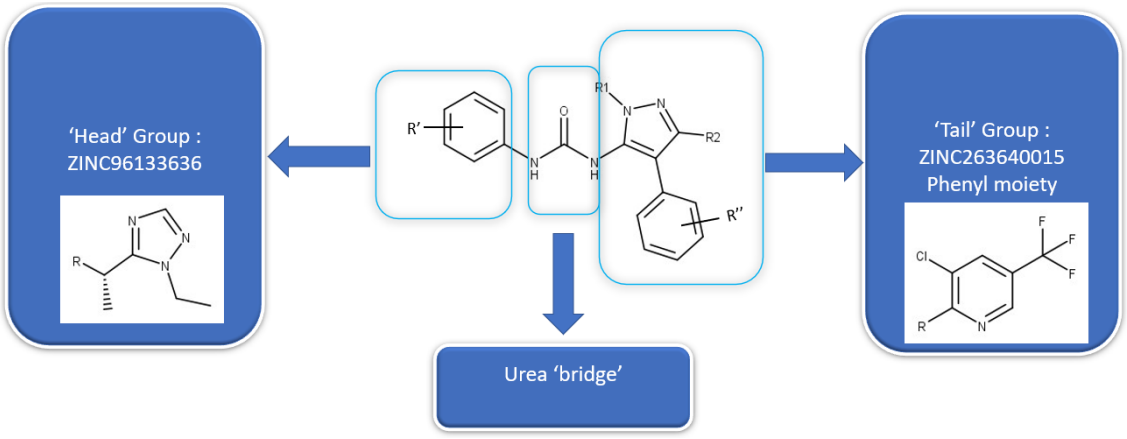


Figure 9. Common structure of pyrazole-urea based PfPRS inhbitors. Both compound ZINC96133636 and ZINC263640015 are modified at the head and tail group, respectively, from the previous SAR study [18]

4. CONCLUSION

The study conducted a hierarchical virtual screening process to identify potential antimalarial candidates through PfPRS enzyme inhibition. This method combines pharmacophore modeling, undesirable moiety filtering, molecular docking, molecular dynamics, and MM-PBSA evaluation, arranged in a sequential manner. Five compounds were discovered from this process, with four possessing a pyrazole-urea scaffold, and the fifth having an isoxazole ring in place of pyrazole. All compounds were tested for antimalarial activity against Plasmodium falciparum 3D7 and exhibited micromolar inhibitory concentrations. Two of the compounds (ZINC 1029449 and ZINC1029453) showed IC50 values of 0.44 and 0.72 µM, respectively. Further studies are still needed to verify whether the compounds inhibit the PfPRS enzyme via allosteric mechanisms.

5. ACKNOWLEDGMENTS

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7. FUNDING

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اكتشاف مثبط إستروزي لأنزيم بروليل–تي آر إن إيه المحتمل عبر الفحص الافتراضي والتجربة في المختبر ضد البلاسموديوم فالسيباروم

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[4]قسم الصيدلة، المعهد الصحي لوزارة الصحة، ماكاسار، إندونيسيا

ملخص

الأهداف: هدفت هذه الدراسة إلى تحديد مركبات مضادة للملاريا الجديدة بناءً على مثبطات موقع مختلف للبروليل–تي–آر–إيه سينثيتيز باستخدام الفرز الافتراضي التسلسلي الهرمي.

المواد والطرق: تم تصميم نموذج الفارماكوفور في البداية، بناءً على بيانات العلاقة بين البنية والنشاط بين عدة مثيلات للبيرازول–اليوريا وقيمتها الإنزيمية IC50. تم تطبيق النموذج المحصل عليه على قاعدة بيانات ZINC15، تليها عملية فلترة المرشحات المتعلقة بشبهات العقاقير والتسمم و PAINS. تم تثبيت المركبات المصنفة باستخدام طريقة التثبيت المصادق عليها ضد إنزيم بروليل–تي–آر–إيه سينثيتيز لـ P. falciparum. تم ترتيب مواضع التثبيت الناتجة بناءً على درجة التثبيت وإعادة التقييم بناءً على معايير الفارماكوفور. تم الحصول على أفضل خمسة مركبات من هذه الخطوة ومن ثم تم تقييمها باستخدام المحاكاة الديناميكية الجزيئية للتحقق من ثباتها وديناميات الروابط الهيدروجينية لأكثر من 50 نانوثانية. تم أيضًا إجراء تحليل MM-PBSA لتقدير طاقة الربط الحرة للمركبات. وأخيرًا، تم التحقق من النشاط الحيوي للمركبات كمرشحات مضادة للملاريا ضد السلالة D7 .3

النتائج: أظهرت النتائج أن جميع المركبات الخمس المحصل عليها من الفرز الافتراضي تمتلك فعالية ميكرومولارية وكان من in vitro.

الكلمات الدالة: مضاد للملاريا، ديناميكا جزيئية، بلاسموديوم فالسيباروم، بروليل–تي–آر–إيه سينثيتيز، فرز افتراضي.

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(2008/23.3/د)

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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