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Jordan Journal of Pharmaceutical Sciences

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INTRODUCTION

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

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

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

Volume 18, 2025

Letter from the Editor-in-Chief

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

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

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

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

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

Best regards

Prof Ibrahim Alabbadi
Editor-in-Chief

CONTENTS

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In vivo Pharmacokinetic Comparison of Oral and Polymeric Nanoparticles Loaded in Transdermal Bilayer Dissolving Microneedles for Nimodipine delivery

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ABSTRACT

Background: Subarachnoid hemorrhage (SAH) is a disease that requires extensive treatment with medication that targets the brain and minimizes systemic adverse effects, preferably with a single daily medication. Nimodipine [NID] offers these properties to be used for this purpose.

Objective: The goal of the study was to accomplish a comparison in the pharmacokinetic parameters of oral nimodipine suspension and transdermal Polymeric Nanoparticles loaded bilayer dissolving microneedles to improve lower oral bioavailability.

Methods: Nimodipine was previously formulated as polymeric nanoparticles (PNPs) characterized by a particle size of 81.78 ± 0.6 nm, a polydispersity index of 0.046 ± 0.01, and a zeta potential of -18.96 mV. These nanoparticles were incorporated into bilayer dissolving microneedle patches (bDMNs) utilizing a casting technique, employing a 10% w/v polyvinyl alcohol (PVA) polymer matrix and 5% glycerin. A total of twelve male white albino rabbits, each weighing approximately 1500 ± 175 g, were randomly allocated into two groups of six animals. One group received an oral dose of nimodipine suspension via oral gavage, while the other group was administered the nimodipine-loaded transdermal bDMNs applied to the skin. The plasma concentration of nimodipine was quantified using reversed-phase high-performance liquid chromatography (RP-HPLC), following the establishment of a spiked calibration curve with plasma samples with the internal standard cilnidipine.

Results: The results displayed mean value of time and concentration needed to achieve the maximum effect were (C_{max}= 42.54 ±3.4 ng/ml, T_{max}= 1 ±0.02 h) for oral and (C_{max}=64.66 ±2.9 ng/ml, T_{max}=0.5±0.01h) for bDMN, respectively approving that the optimized transdermal bDMN exhibited higher plasma concentration with T_{max} lower than oral route, achieving (1.9) fold rise in the calculated relative bioavailability.

Conclusions: The transdermal bDMNs could offer a promising and effective method for NID delivery to improved lower oral bioavailability by enhancing the delivery through skin.

Keywords: Subarachnoid hemorrhage, transdermal, bilayer, bioavailability.

INTRODUCTION

Regardless of their post-ictus neurological condition, NID can enhance neurological outcomes by lowering the prevalence and severity of ischemia impairments [1]. In 1988, the FDA first gave its approval for clinical uses in treating SAH [2]. The parenteral NID formulation has a high absolute bioavailability (100 %) but it causes painful administration with the need for hospitalization resulting in low efficacy. Oral administration of the NID using current dosage form (tablets and capsule) has low plasma concentrations and required a high daily doses to achieve its therapeutic effect due to the extensive first-pass metabolism [3]. Additionally, drawbacks such low drug solubility, repeated daily oral doses were required ending with insufficient bioavailability (13-30%) and undesirable side effects.

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The study described using a combination technique involving bilayer dissolving microneedles (bDMNs) and Nano encapsulation for delivering a lipophilic model systemically via the skin [1]. As a result, a transdermal delivery bDMN-loaded was developed to overcome skin barrier stratum corneum (SC) while PNPs compromise a number of features, including increasing lower solubility, targeted drug delivery, regulate the skin permeability, prevention of incorporated drug from degradation in the biological environment, consequently reduced dose and side effects [4, 5].Therefore, selective manipulation of size, chemical composition, shape, internal structure, surface charge, and combination approaches allows for controlling drug release from PNPs.

There is consensus in the literature that physical approaches such as microneedles are necessary for PNPs to enhance the permeation through the SC [6]. The drug is usually released and carried to deeper layers of the epidermis by the hair follicles. The transporting PNPs via bDMN beyond SC, achieved by creating microsized channels within skin, thereby increasing the translocation of NPs as reservoirs of drugs deeper to skin [7].

As a results, transdermal drug delivery is an attractive option that deliver NID systemically achieving avoidance of first pass metabolism in addition to the advantages of bDMNs such as being noninvasive, painless self-administered dosage form .This study developed new bDMNs formulations of NID for transdermal application to improve lower oral bioavailability via preventing presystemic metabolism and lowering side effect of NID [4, 7].

The primary objective of this study was comparing the pharmacokinetic parameters of oral NID suspension with transdermal PNPs loaded bDMNs, to enhance oral bioavailability.

MATERIALS AND METHODS

Materials

Nimodipine (CAS Number: 66085-59-4) was purchased from Zhejiang Shenzhou pharmaceutical Co., LTD, China, soluplus was purchased from Sigma-Aldrich®, PVA and PVP was purchased from HI Media Laboratories, USA. HPLC grade Acetonitrile, HPLC grade Methanol and all other chemicals were obtained from Merck chemical division, Mumbai. Ethanol was purchased from Honeywell International Inc. USA.

Preparation of NID-NP-DMN

Nimodipine was previously formulated, characterized, and optimized, as shown in Table 1. The formulation contained one dose of the drug mixed with Soluplus (1:8, w/w), dissolved in 3 mL of ethanol, which was then added to an aqueous phase containing 0.25% PVP K15. The mixture was stirred until a uniform distribution was achieved, resulting in particles with a size of 81.78 ± 0.6 nm. Subsequently, the casting technique was employed to fabricate bDMNs (array 15 × 15, 500 µm height) using 10% PVA as the polymeric solution [8,9].

Table 1. Composition of NID-PN-DMN

Ingredients	Amount
Nimodipine	30mg
Soluplus *	240mg
Absolute ethanol	3ml
PVPK15**	0.075mg
D.W***	27ml
Glycerin	5%v/v
PVA	10%w/v
Soluplus®= polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol graft copolymer **PVPK15=polyvinylpyrrilodine K15, ***DW= deionized water	

Study Design

This study utilized twelve male white albino rabbits, which were randomly divided into two groups, each consist of six rabbits (n=6), with an average weight of 1500±175 g. The rabbits were confined in the animal facility of the Research Centre for Cancer Research and

Medical Genetics, Baghdad, Iraq, under a constant room temperature of 25°C±1°C, a 12h light/dark cycle for 7 days prior to the experiment to be acclimatized to laboratory conditions [10].

Dosing Regimen

Before administration, rabbits were subjected to food fasting for12 hr. The animals dose (3.08mg/kg) was calculated depending on human dose (1mg/kg), according to equation (1), the rabbits (1500 ± 175g) given oral dose of the NID suspension using an oral gavage as the control group while the second groups were administered NID by inserted transdermal bDMNs into the full thickness of dorsal skin after removing their back hair using an electric shaver [2].

$$\boldsymbol{HED}\left(\frac{mg}{kg}\right) = \boldsymbol{AED}\left(\frac{mg}{kg}\right) \times \frac{AnimalKm}{Human\ Km} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Eq.1$$

Where, HED is the human equivalent dose, AED is the animal equivalent dose, and Km is the conversion factor (for human adults=37, and for rabbits =12) so the ratio will be 3.0 [3,4] .During administration, both groups were administered ketamine (Ketamine 10%, Alfasan Woerden, Holland) at a dose of 35 mg/kg, and xylazine (XYL-M2, VMD® HogeMauw 900 pharma, Belgium) at a dose of 5 mg/kg , the study concentrated on 48 h and divided the times of sample collection [7].

In vivo pharmacokinetic studies

The study was concentrating to evaluate the drug delivery of NID transdermaly through the application of NID-PNP Loaded bDMNs. Each sampling was timed, and a single dose was administered to both groups to assess the relative bioavailability of the oral NID suspension and transdermal NID-PNP-bDMN. A total of 1 mL of blood samples were taken from heart before administration (0 min) and at 5, 10, 20, 30, 45, 1hr, 2, 6, 12, 24 and 48 hr., post injection, was acquired from the myocardium through piercing at predefined time intervals .An EDTA-treated tubes were used in order to collect blood samples from the rabbits, which were then promptly separated. Plasma samples were obtained by centrifuging the blood samples (Hettich Zentrifugen EBA 20, Germany) at a speed of 3000 rpm for a duration of 15 min. Plasma samples were obtained from the supernatant liquid fraction, transported to Eppendorf tubes, and stored at -20 °C for analysis .All samples were sheltered from exposure to light to avert any potential photo-degradation of NID in the plasma [11].

A confirmed approach was used to evaluate the samples of plasma by using reversed high-performance liquid chromatography (RE-HPLC). Maximum concentration (C_{max}) and time to reach C_{max} (T_{max}) were also determined. The AUC_{0-48} were determined by calculating the integral of the plasma concentration-time curve from time 0 to 48 hrs. by applying Trapezoidal rule. The relative bioavailability value (F) was calculated using equation 2 [12, 13].

$$\boldsymbol{Relative\ Bioavailability} = \frac{AUC\ NID-PNP-MN \times doseof\ \ NID\ suspension}{AUC\ NID\ suspension \times dose\ of\ NID-PNP-MN} * \mathbf{100} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Eq.2$$

Analytical method

Plasma concentration of NID was determined using RE- HPLC analysis according to the procedure that was developed and validated in term of linearity, precision, accuracy, lower limit of detection, and lower limit of quantification by ***Rajani B***. in estimating the NID in tablet dosage form as shown in Table.2 [14,15].

Table 2. Chromatographic Conditions

Parameter	Condition
Mobile phase	PB:Acetonitril(30:70)
Diluent	Methanol :DW(80:20)
Column	C18(4.6 x150mm, 5µ)
Column temperature	25^0C
Flow rate	1 ml/min
Retention time	3.79min
Run time	20 min
Wavelength	236nm
Injection volume	10 µL

Calibration Curves

Standard solutions containing NID were used to spike control plasma to create calibration standards at concentrations of 0.1, 0.5, 2.5,5,10, 20 and 60 ng/ml. A constant concentration 5ng/ml of cilnidipine as internal standard (IS) was added to all assay tubes. Zero-concentration plasma samples containing only the IS were included in each run. In addition, a plasma blank sample (without IS) was analyzed in each cycle [16].

Validation Method

Linearity and limits of detection and quantitation

The proposed method's linearity was confirmed by constructing calibration curves at (0.1 - 60 ng/ml) NID concentrations and plotting their relative peak area (against their respective concentrations using a linear least squares regression analysis, using cilnidipine as the internal standard [17].

Precision and Accuracy

Precision measured by calculating the relative standard deviation of intra-day repeatability and inter-day reproducibility while accuracy determining the percent recovery using three different concentrations as described by ***Rajani B*** [14,15].

Sample Extraction

To prevent photodegradation of NID, all experimental procedures—including plasma collection, sample preparation, and instrumental assays—were performed under dim yellow light. Extraction was carried out according to the method developed by Nascimento DFd et al. Briefly, 300 µL of plasma was mixed with 25 µL of the internal standard (cilnidipine, 5 ng/mL) and vortexed for one minute. Subsequently, 1000 µL of a hexane/ethyl acetate mixture (1:1, v/v) was added, followed by five minutes of vortex mixing. The samples were then centrifuged at 2000 g for five minutes at 37 °C. The upper organic layer (800 µL) was carefully collected, transferred to clean tubes, and dried in a vacuum desiccator (Christ Company, Germany) at 37 °C. The residue was reconstituted with 150 µL of acetonitrile (ACN) and vortex-mixed for one minute. The resulting solutions were transferred to microvials, sealed with caps, and placed in an autosampler rack. Finally, 10 µL aliquots were injected into the chromatographic system [17,18]

Statistical analysis:

The results were presented as mean values with their standard deviation (±SD; n=3). A difference was judged statistically (using T-test) significant or not if the P-value was < 0.05 using Prism GraphPad 8.4.3 Software. The pharmacokinetic parameters, C_{max}, T_{max}, and AUC_{0-48}, were calculated by means of PK solver 2.0[19]

RESULTS AND DISCUSSION

Validation Method

Linearity and limits of detection and quantitation

Least squares regression calibration curves were created by plotting the peak area ratios of NID to IS against nominal concentrations. The correlation coefficient (R^2) was 0.99994 over the point out that the calibration curve obeys Beer's law within the range of concentration range of 0.1–60 ng/ml used [20].

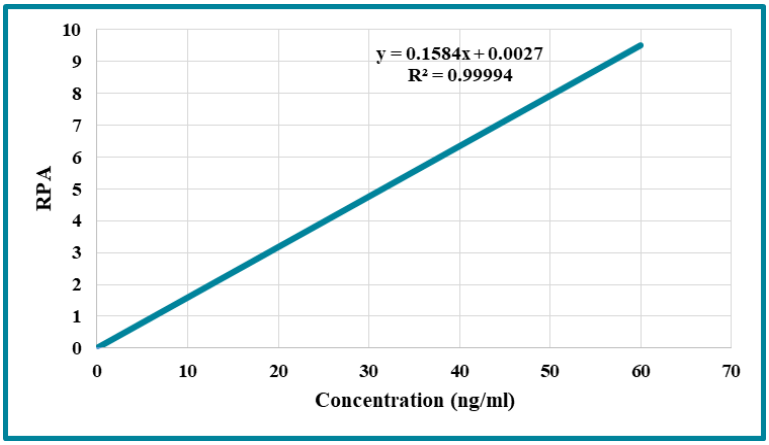

Figure 1. Calibration curve of NID with IS as an internal standard in the mobile phase (Potassium dihydrogen phosphate buffer: Acet) (30:70, v/v). (Error bars= SD, n=3)

The sample's chromatogram exhibited a good separation attained within 20 min using the conditions described. Sharp, symmetrical, and well-resolved peaks were detected for NID and IS. The elution order and the retention times for spiked plasma, NID besides IS were 1.47±0.002min, 3.79± 0.004 min and 6.18 ± 0.005 min respectively (Figure 2).

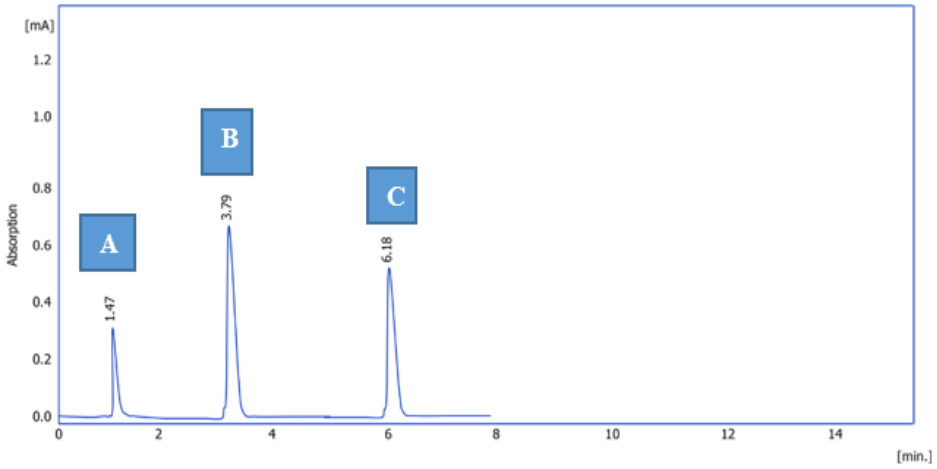

Figure 2. Chromatogram of known concentration of NID spiked with plasma (A), NID (B), and the internal standard (C) (n=3)

The HPLC method was considered to calculate plasma concentration of NID and method was valid (linearity R^2 = 0.9994) and both limits of detection and quantification (LOD and LOQ) were determined giving to the following equations as per ICH guidelines [21].

LOD = 3.3* ẟ/S ……Eq.3

LOQ = 10* ẟ/S ……Eq.4

Where ẟ is the standard deviation of y-intercept of calibration curves and S is the mean of slope of calibration curve [1, 21]. The lower limit of quantification (LOQ) for NID was 0.12 ng/ml, the lowest concentration on the calibration curves and the LOQ was 0.37 ng/ml.

Regarding precision and accuracy, the study revealed validation parameters that were consistent with results mentioned by ***Rajani B***. According to the standard guidelines, the proposed analysis method is precise since the mean values of %RSD for all were less than 20% and the recovery was 97.66% which is within range of 99%-101%indicating that the proposed method was accurate [14].

***In-vivo* Pharmacokinetics Results**

The *in-vivo* pharmacokinetics study achieved by administering one equivalent dose of NID oral suspension, and transdermal NID-NP-bDMNs followed by plasma sample collection to attain C_{max}, T_{max} and AUC as displayed in (Table 3) and plasma profiles (Figure 3) where the resulted average relative bioavailability of transdermal NID-NP-DMNs was 1.9 fold higher than oral NID suspension.

Table 3. Pharmacokinetics Parameters of Nimodipine

Pharmacokinetic parameters	NID oral suspension	Optimized transdermal NID-NP-bDMNs
C max (ng/ml)	42.54 ± 3.4	64.66 ± 2.9
T max (hr.)	1± 0.02	0.5 ± 0.01
AUC $_{0—48}$ (ng.h/ml)	27230.08±25.4	51947.56±18.3

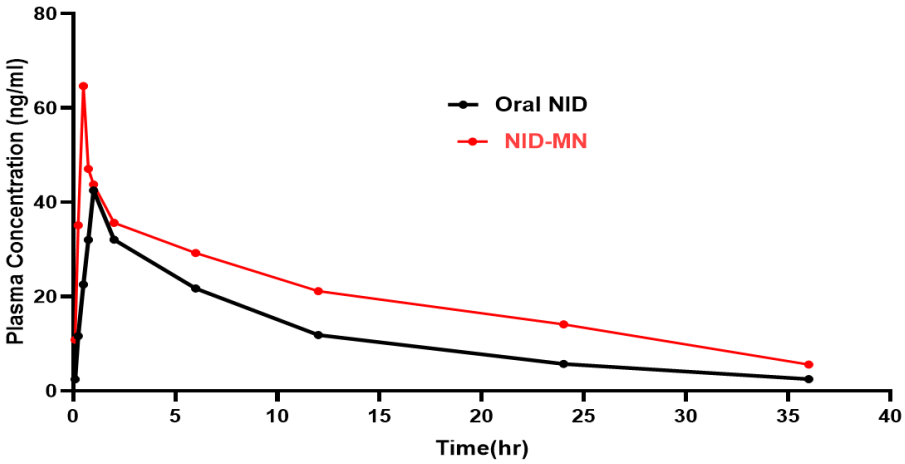

Figure 3. Comparative *in vivo* plasma profile of NID oral suspension and transdermal NID NP-bDMNs formula

Concerning the Nanocarriers, including polymeric nanoparticles, are specifically created to carry therapeutic drugs that exhibit distinct physicochemical properties and are composed of different polymer ratios. Extensive research has been conducted on dissolving microneedles to enhance the transdermal delivery of medicinal substances ended with several advantages; first, it effectively penetrates SC that acts as the primary obstacle to drug diffusion and second, to avoid the extensive first pass metabolism that resulting in rapid decline in plasma concentrations following oral NID administration [18, 23].

Statistical analysis revealed that (C_{max}= 42.54 ±3.4 ng/ml, T_{max}= 1 ±0.02 h) for oral NID while (C_{max}=64.66 ±2.9 ng/ml, T_{max}=0.42±0.01h) for b DMN, respectively. Plasma concentrations of NID are significantly higher (P= 0.022) with lower T_{max} following DMNs administration in contrast to low and inconsistent plasma levels of NID subsequent oral administration [24].

As a results, the pharmacokinetic analysis exhibited that the optimized transdermal NID-NP-bDMN had a 1.9 fold rise in the relative bioavailability compared to oral NID suspension. Additionally, an important factor that significantly enhances the lower oral bioavailability is the reduction in particle size with formulation of nanoparticles that leads to an increase the surface area of the NID, hence boosting the dissolving of medications that are poorly water soluble[2,21].

CONCLUSION

Nimodipine as an effective treatment for the avoidance of SAH, nevertheless the mechanism of action and dose limiting hypotension persist foremost areas of uncertainty. Recent advances in delivery have confirmed safety of drug delivery with less hypotension and significantly higher concentration at the target organ.

Results demonstrate that the convenient, painless, and less invasive bilayer dissolving microneedles employed as an alternative to injection and oral administration through enhanced transdermal delivery of NID offering an improvement of lower oral bioavailability, reduced side effect from intravenous administration and improved patient compliance.

The formulated NID-NP-loaded bDMN has a much higher relative bioavailability compared to the oral NID. Consequently, it is deemed as a more advantageous dosing form for the administration of NID in the treatment of SAH.

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Ethics Statements

The animal studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC), College of Pharmacy, University of Baghdad, Iraq. Authorization was granted for the in vivo experiments on rabbits under protocol number RECAUBCP 2720236.

Author Contribution

Asmaa. M.R. Data collection, investigation, methodology, writing—original draft preparation. Mowafaq. M.G. Project administration, supervision, writing—review and approval of the final manuscript.

Abbreviations

Abbreviations	Meanings
AUC	area under curve
C	Celsius
Cmax	Maximum concentration
DMN	Dissolve microneedle
g	Gram
hr.	Hour
IS	internal slandered
Kg	kilogram
LOD	Limit of detection
LOQ	Limit of quantification
mg	milligram
µ	microliter
-min	minute
-ml	Milliliter
-mm	millimeter
-ng	Nano gram
NID	nimodipine
NPs	nanoparticles
PVA	Polyvinyl alcohol
PVPK15	polyvinylpyrrolidone
RE-HPLC	Reversed high-performance liquid chromatography.
-rpm	Round per minute
SAH	subarachnoid hemorrhage
SC	stratum corneum
Tmax	Maximum time
V/V	Volume by volume
W/V	Weight by volume

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مقارنة داخل الجسم الحي بين التوصيل الفموي والتوصيل عبرالجلد لجسيمات النيمودبين البوليمرية النانوية المحملة بابر دقيقة مذابة ثنائية الطبقات

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ملخص

الخلفية: نزيف تحت العنكبوتية (SAH) هو مرض يتطلب علاجا مكثفا بالأدوية والتي تستهدف الدماغ وتقلل من الآثارالجانبية الضارة ، ويفضل أن يكون ذلك باستخدام دواء يومي واحد. يقدم نيموديبين هذه الخصائص لاستخدامها لهذا الغرض

الهدف: هدفت الدراسة إلى مقارنة معايير الحركية الدوائية لمعلق نيموديبين الفموي مع الإبر الدقيقة الذائبة المحملة بجسيمات نانوية بوليمرية عبر الجلد من اجل زيادة التوافر الحيوي الفموي المنخفض.

الطريقة: تم تصييغ النيموديبين سابقا على شكل جسيمات نانوية بوليمرية ، تتميز بحجم جسيمات يبلغ 81.78 ± 0.6 نانومتر، ومؤشر تعدد تشتت يبلغ 0.046 ± 0.01، وجهد زيتا يبلغ −18.96 ملي فولت. وقد صب هذه الجسيمات النانوية في رقعات إبر دقيقة مذابة ثنائية الطبقات باستخدام تقنية الصب، باستخدام بوليمر البولي فينيل الكحول بنسبة 10% وزن/حجم و5% جلسرين .وُزّع اثني عشر أرنبًا أبيض ، ذكرًا من نوع ألبينو، يزن كل منها حوالي 1500 ± 175 غرامًا، عشوائيًا على مجموعتين من ستة حيوانات. تلقت إحدى المجموعتين جرعة فموية من معلق النيموديبين عن طريق التغذية الأنبوبية، بينما أُعطيت المجموعة الأخرى رقعات إبر دقيقة مذابة ثنائية الطبقات عبر الجلد، محملة بجسيمات نيموديبين النانوية . تم تحديد تركيز نيموديبين في البلازما باستخدام كروماتوغرافيا السائل عالية الأداء في الطور العكسي (RP-HPLC)، بعد إنشاء منحنى معايرة مدبب مع عينات البلازما ذات المعيار الداخلي للسيلينيديين.

النتائج: أظهرت النتائج أن الوقت والتركيز اللازمين لتحقيق أقصى تأثير كانا C_{max} = 42.54 ±3.4نانوغرام/مل، T_{max}=1 ± 0.2 ساعة للإعطاء الفموي، و C_{max} = 64.66 ± 2.9 نانوغرام/مل، T_{max}=0.5 ± 0.1 ساعة للإعطاء عن طريق الجلدعلى التوالي. لقد أظهر تحليل الحركية الدوائية أن تركيبة الابر الدقيقة المذابة ثنائية الطبقة والمحملة بالجسيمات النانوية عبر الجلد شهدت زيادة في التوافر الحيوي النسبي بمقدار 1،9 مرة مقارنةً بمعلق النيموديبين الفموي.

الخلاصة: يمكن أن يوفر التوصيل عبر الجلد باستخدام أن تقنية الابر الدقيقة المذابة ثنائية الطبقة والمحملة بالجسيمات النانوية طريقة واعدة وأكثر فعالية لتوصيل الدواء.

الكلمات الدالة: النزف تحت العنكبوتية، عبر الجلد، الطبقة المتقرنة ، ثنائي الطبقة ، التوافر الحيوي.

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Control-Release Polyethylenimine-Modified Fibroin Nanoparticles As A Potential Vehicle for the Oral Delivery of Quercetin

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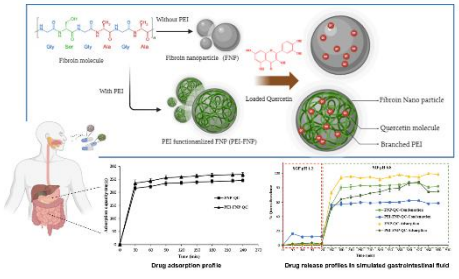
ABSTRACT

Polyphenolic compounds are a big class of chemicals employed in numerous biomedical applications. However, these compounds are susceptible to degradations, especially in the varied gastrointestinal pH, which hinders their use in oral administrations. Thus, this work developed fibroin nanoparticles (FNP) and polyethylenimine-modified FNP (PEI-FNP) to orally protect and deliver quercetin (QC), a model polyphenol. The particles were formulated using two distinct methods: adsorption and co-condensation. Both formulas showed appropriate physicochemical properties for oral administrations, including nano-sizes (~700 nm for FNP-QC and ~200 nm for PEI-FNP-QC), narrow size distribution (polydispersity index < 0.3), adjustable zeta potentials (~-20 mV for FNP-QC and ~+25 mV for PEI-FNP-QC), enhanced QC aqueous solubility to 2-3 times, and observable chemical interactions (hydrogen bonding and ionic interactions) between QC and fibroin/PEI. Moreover, depending on the formulation process and particle compositions, the particles possessed moderate QC entrapment efficiency (35-75%), smooth/rough surfaces, and rapid drug adsorption followed models including Langmuir and Dubinin-Radushkevich isotherms, as well as pseudo-second-order kinetics. Interestingly, in the mimicked oral condition, the particles can protect QC from the gastric condition at pH 1.2, with less than 20% QC release, while sustaining its release in the intestine at pH 6.8, with the release rates that could be favorably controlled by varying the formulation methods and/or PEI functionalization. In summary, the FNP and PEI-FNP demonstrated much potential as release-controllable delivery systems for oral administrations of polyphenolic compounds.

Keywords: Fibroin; Polyethylenimine; Nanoparticles; Quercetin; Oral delivery

GRAPHICAL ABSTRACT



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1. INTRODUCTION

Polyphenolic compounds are bioactive substances prevalent in almost every medicinal plants, possess numerous potential therapeutic efficacies [1–3]. Despite their benefits, the effective utilization of polyphenols in oral administration is significantly hampered due to their susceptibility to degradation in varying pH conditions, particularly within the gastrointestinal tract [4]. This instability reduces their bioavailability, limiting their therapeutic efficacy when consumed orally. Previous studies have attempted to address these challenges by developing drug delivery systems, namely fibroin micro-/nanoparticles (FNP), to encapsulate, protect, and sustain the release of polyphenols in different plant extracts of guava [5] and wedelia [6]. These research demonstrated that FNP could highly protect these polyphenolic compounds from degradation while preserving their bioactivity. Nevertheless, no research has focused on the FNP ability to orally deliver the polyphenols in the gastrointestinal tract.

To bridge this gap, the current study focuses on the development of FNP, together with its functionalization counterpart, polyethylenimine-modified FNP (PEI-FNP), to encapsulate quercetin (QC), a model polyphenol, for oral application. QC is a popular plant flavonoid found in various vegetables and fruits, which has numerous pharmacological effects, such as reducing the inflammatory response (regulating eicosanoids biosynthesis), reducing the low-density liporotein oxidation (preventing atherosclerotic plaque formation), and regulating the enzymatic activities of ornithine carboxylase, calmodulin, or protein kinase [7,8]. However, QC application in the pharmaceutical area is constrained by its poor water solubility, low permeability, instability in the gastrointestinal environment, and susceptibility to extensive first-pass metabolism [9–11]. Moreover, QC interacts with various dietary components and its stability is influenced by the pH and temperature conditions in the gastrointestinal tract [12]. To this end, drug delivery systems such as liposomes [13,14], lipid-nanocapsules [15], or inorganic materials such as silica microspheres [16,17], have been explored to improve QC solubility and bioavailability. In this context, proteins have a protective effect and resist degradation of QC, as hydrophobic interactions with proteins are responsible for stabilizing QC [18,19]. Thus, protein-based delivery systems such as FNP and PEI-FNP could be a potential approach for QC oral delivery.

Fibroin is the main protein in the silk fiber core, recognized by the US FDA as a biomedical material [20,21]. Fibroin has excellent biological properties such as water solubility (in its silk I form) [22,23], slow biodegradation [24,25], non-toxicity and biocompatibility [26–28], making it an outstanding biomaterial. As such, fibroin usages in oral drug delivery has increasingly received a lot of attention [29–31]. The unique fibroin structure of anti-parallel amino acid chains with the interactions of intramolecular and intermolecular hydrogen bonds along with van der Waal forces and hydrophobic bonds, providing a stable 3-dimensional structure, thereby increasing fibroin's flexibility, making it stable in a wide pH range and other degrading factors [32,33]. Additionally, fibroin has the ability to adhere to mucous membranes [34], making FNP to adhere tightly to intestinal epithelial cells, consequently enhance the encapsulated drug oral bioavailability. At the same time, fibroin degradation in the digestive tract usually takes place slowly (about a few weeks), giving FNP enough time to perform their effects [35]. Last but not least, the modification of FNP with PEI, a positively charged polymer commonly used in gene transfer with low cytotoxicity [36], through the ionic interactions, could increase the particle rigidity, drug entrapment efficiency, and controllable release efficiency [37,38].

Ultimately, the main objective of the present work was to focus on the development and usage of FNP and PEI-FNP for the oral delivery of QC. This study aims to fill a significant research gap in the field of polyphenol bioavailability and oral drug delivery systems, providing a novel approach to solve the challenges allied with the gastrointestinal stability and bioavailability of polyphenolic compounds.

2. MATERIALS AND METHODS

2.1. Materials

Cocoons of the *Bombyx mori* silkworm were gathered from Nam Dinh, Vietnam, and fibroin was extracted and purified utilizing the standard process [30]. The source of QC and PEI (branched, molecular weight of 25 kDa) was imported from Sigma-Aldrich, Singapore. Ethanol (99.5%) and other chemicals were supplied by general chemical companies and were of at least reagent grade or higher.

2.2. Preparation of the FNP and PEI-FNP

The QC-loaded FNP (FNP-QC) and QC-loaded PEI-FNP (PEI-FNP-QC) were prepared using two different methods: adsorption and co-condensation. For the co-condensation method, 1 mL of the fibroin aqueous solution (8 µg/mL) was mixed with 1 mL ethanol comprising 5 mg QC, without/with 1 mL PEI solution (1% w/v, pH 7.0), to yield the PEI-FNP-QC and FNP-QC, respectively. The mixture was maintained for 24 h at 4°C, cold centrifuged (18,000 rpm, 30 min), and the resulting particles were washed thrice with water. The blank FNP and blank PEI-FNP were also prepared similarly [30]. All particles were freeze-dried (-55°C, 72 h) and stored at 4°C for further investigations [38]. The free/unencapsulated QC in the centrifuge supernatant was measured by UV-Vis spectroscopy at 370 nm, using a calibration curve (range 0-32 µg/mL, y = 0.0723x - 0.0093, R^2 = 0.9995), and the QC entrapment efficiency (EE%) was calculated according to **Eq. (1)** [31].

$$EE\% = \frac{5 - Amount\ of\ unencapsulated\ QC\ (mg)}{5}\ x\ 100 \quad (1)$$

For the adsorption method, the blank FNP or blank PEI-FNP (0.015 g) were dispersed in 50 mL of QC ethanolic solution (100 µg/mL) for 4 h. To monitor the adsorption process, 1 mL of the dispersion was taken out every 30 min, with medium refilled. The samples were then centrifuged (18,000 rpm, 3 min), and the centrifuge supernatants were UV-Vis spectroscopic analyzed at 370 nm to evaluate the unadsorbed QC. The QC adsorption efficiency (%) was calculated according to **Eq. (2)**, where Ce is the QC equilibrium concentration (µg/mL).

$$QC\ adsorption\ efficiency\ (\%) = \frac{100 - C_e}{100}\ x\ 100 \quad (2)$$

2.3. Characterizations of the FNP and PEI-FNP

Particle size, polydispersity index, and zeta potential

The mean particle size, size distribution (polydispersity index, PI), and zeta potentials were measured by the dynamic light scattering (DLS) and phase analysis light scattering (PALS) technique, respectively, using ZetaPALS® analyzer (Brookhaven Instrument Corporation, USA) installed with a helium-neon laser diode (35 mW, 632.8 nm). The particles were diluted with water until a count rate of 500-600 kcps, and the measurements were conducted in triplicate at 25°C.

Particles morphology

Scanning electron microscope (SEM, Carl Zeiss, Germany) was utilized to illustrate the particle morphology. The particles were re-dispersed in water and diluted until a count rate of 400 kcps and the dispersions were dropwise added onto 100-nm plastic discs, which were mounted on a metal base, followed by gold coating, and observed by SEM.

Particles structure

The particle structures and chemical interactions were determined using Fourier-transform infrared spectroscopy (FT-IR, Jasco 6300, Japan), using the KBr tablet technique. The spectra were acquired in a 4000-400 cm^{-1} wavenumber range at a resolution of 4.0 cm^{-1}.

Drug solubility

The aqueous solubility of the free/pure QC, QC in FNP-QC, and QC in PEI-FNP-QC, was tested in phosphate buffer saline (PBS). The samples were dispersed in PBS, stirred overnight (200 rpm, 25°C), cold centrifuged (18,000 rpm, 30 min), and the supernatant was UV-Vis measured at 376.5 nm. The QC solubility was determined as the highest concentration of QC in PBS,

with a QC standard curve in PBS (y = 0.0684x - 0.0037, R^2 = 0.9993).

2.4. Isotherm and kinetics of QC adsorption

The QC adsorption process onto the FNP and PEI-FNP was evaluated utilizing the standard isothermal and kinetics models. The Langmuir model (**Eq. (3)**) describes whether the adsorption process is monolayer or multilayers. The Freundlich model describes a physical type of adsorption occurring in multilayer, with the assumption that the adsorption sites are heterogeneous (**Eq. (4)**). The Dubinin-Radushkevich model (**Eq. (5)**) assesses the adsorption nature of physical or chemical adsorption. The pseudo-first-order kinetics (**Eq. (6)**) and pseudo-second-order kinetics (**Eq. (7)**) show the adsorption rates and properties of the QC onto the particles.

$$\frac{q_e}{q_m} = \frac{K_L C_e}{1 + K_L C_e} \quad (3)$$

$$q_e = K_F C_e^{1/n} \quad (4)$$

$$\ln q_e = \ln q_m - \beta\varepsilon^2 \quad (5)$$

$$\ln(q_e - q_t) = \ln(q_e) - k_1 t \quad (6)$$

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (7)$$

where q_e, q_m, q_t are the QC equilibrium adsorption capacity, the maximum adsorption capacity, and the adsorption capacity at time point t (mg/g), respectively; C_e is the FNP/PEI-FNP concentration (mg/L); β is the adsorption energy constant; K_L is the Langmuir constant; K_F is the Freundlich constant; n is the number demonstrating the degree of linearity between the adsorbate and the adsorption process; ε is the Polanyi potential energy; k_1 and k_2 are the pseudo-first-order and pseudo-second-order kinetics rate constant.

2.5. In-vitro QC release in simulated oral condition

The FNP-QC and PEI-FNP-QC were evaluated for their ability to release QC at 37°C in the mimicked gastrointestinal condition, utilizing the standard shaking method. Firstly, the FNP-QC and PEI-FNP-QC were dispersed in 10 mL HCl (pH 1.2), which simulates gastrointestinal juice, for 2 h. Then, the FNP-QC/PEI-FNP-QC were subjected to 40 mL PBS (pH 6.8), which simulates intestinal fluid, for an additional 6 h. Every 30 min, 1 mL dispersion was taken out, with medium refilled, centrifuged at 18,000 rpm for 3 min, and the QC released was measured by UV-Vis spectroscopy method. The absorbance was determined at 368.5 nm in the HCl medium (y = 0.0553x - 0.0048, R^2 = 0.9985, range 0-10 µg/mL), and at 376.5 nm in the PBS medium (y = 0.0684x - 0.0037, R^2 = 0.9993, range 0-10 µg/mL). The QC release (%) was determined by **Eq. (8)**, with C_t and C_i are the released QC concentrations at the time t and i, V_o is the total medium volume, M_o and M_i are the QC initial and the QC withdrawal amount at the time i.

$$\text{QC release (\%)} = \frac{C_t V_0 + \sum_1^{t-1} C_i}{M_0 - \sum_1^{t-1} M_i} \, x \, 100 \quad (8)$$

3. RESULTS AND DISCUSSIONS

3.1 Preparation of the FNP and PEI-FNP

In this study, the FNP-QC and PEI-FNP-QC were formulated using the simple one-pot preparations employing the co-condensation or adsorption technique. Then, all particles were characterized in terms of sizes, PI, zeta potentials, morphology, chemical interactions, and QC solubility. Finally, the in-vitro QC release profiles in the oral simulated condition were investigated. The particles were successfully prepared and characterized, discussed in the next sections.

3.2. Characterizations of the FNP and PEI-FNP

Firstly, both the blank and QC-loaded particles were successfully prepared with nano-sized (150-700 nm) and narrow size distribution (PI < 0.3) (**Table 1**). The PEI-FNP showed statistically smaller sizes than those of the FNP, which was due to the tightening and rigidifying effects based on ionic interactions between the PEI (positive

charge) and the fibroin molecules (negative charge), in agreements with previous studies [30,38]. When being loaded with QC, the particles sizes increased, possibly due to additional interactions (i.e., hydrophobic interactions, hydrogen bonding, and ionic interactions) between the QC and fibroin/PEI, consequently enlarging the particles [6]. These interactions are discussed in the FT-IR section.

Secondly, the zeta potentials of PEI-FNP shifted to positive values, indicating the presence of PEI, whereas the FNP showed negative values of the fibroin inherent negative charge. This fact expands the versatility of FNP in biomedical applications [38,39]. Moreover, the incorporation of QC into the particulate systems slightly reduced the zeta potentials to a more negative value (i.e., from -15 mV of blank FNP to -22 mV of FNP-QC; from +33 mV of blank PEI-FNP to +26 mV of PEI-FNP-QC). This was because the QC has five pKa of 7.17, 8.26, 10.13, 12,30, and 13.11 [40], making QC mostly stays in the anionic form at neutral pH, consequently shifted the system zeta potentials to be more negative.

Table 1. Particle size, PI, zeta potential, and QC EE% of the FNP-QC and PEI-FNP-QC, together with the blank counterparts (n = 3).

Formula	Size (nm)	PI	Zeta potential (mV)	QC entrapment efficiency (%)	
				Co-condensation	Adsorption
Blank FNP	328.1 ± 13.4	0.16 ± 0.02	-15.5 ± 1.1	-	-
Blank PEI-FNP	176.2 ± 10.2	0.11 ± 0.03	+33.7 ± 2.4	-	-
FNP-QC	680.4 ± 24.7	0.12 ± 0.02	-22.7 ± 1.2	35.8 ± 2.6	67.9 ± 2.2
PEI-FNP-QC	219.8 ± 20.3	0.13 ± 0.02	+26.7 ± 2.9	55.9 ± 5.3	75.5 ± 2.9

Regarding the QC EE%, in the preliminary experiments, the initial QC loading amounts were varied (1 mg, 5 mg, and 10 mg) in both formulation methods. The results showed that at high QC amount of 10 mg, the particles were unstable, easy to aggregate to from micron-size clumps, whereas at lower QC amounts of 1 mg and 5 mg, the nanoparticles were achieved. Moreover, no significant differences were noted between the formulas with 1 mg and 5 mg QC. Thus, to maximize the drug loading amount, we selected the initial QC amount of 5 mg. Both co-condensation and adsorption techniques yielded a moderate EE% of 30-70%, depending on the entrapment method (**Table 1**). It was found that the adsorption method had a higher EE% than that of the co-condensation method for both formulations. This was due to disparities in drug molecule behaviors concerning the two methods. Regarding the co-condensation technique, the drug molecules dissolved in ethanol were co-condensed with fibroin during the particle formation [41], consequently, the QC molecules were encapsulated in the particle mainly in the crystalline form (proven in the SEM images, with the QC crystals presented on the particle surfaces (**Figure 1**)). Due to the moderate solubility of QC in ethanol (4 mg/mL at 37°C [42]), a part of the drug molecules stayed in the amorphous form and was not co-precipitated, thereby reducing the QC EE%. Besides, in the adsorption process, all drug molecules (100%) were in molecular form and can freely interact with FNP and PEI-FNP. As a consequence, more QC could be adsorbed and absorbed onto and into the particles in its amorphous form, thus increasing the EE% [30]. Interestingly, the PEI-FNP provided higher QC EE% than that of FNP for both adsorption and co-condensation methods. This was because of the additional ionic interactions between the PEI (positive charge) and QC (negative charge), which make more QC molecules being encapsulated into the particles [38,43]. On the other hand, both the fibroin and QC had negative charges, thus the repulsion forces decreased the EE% of the FNP-QC formula.

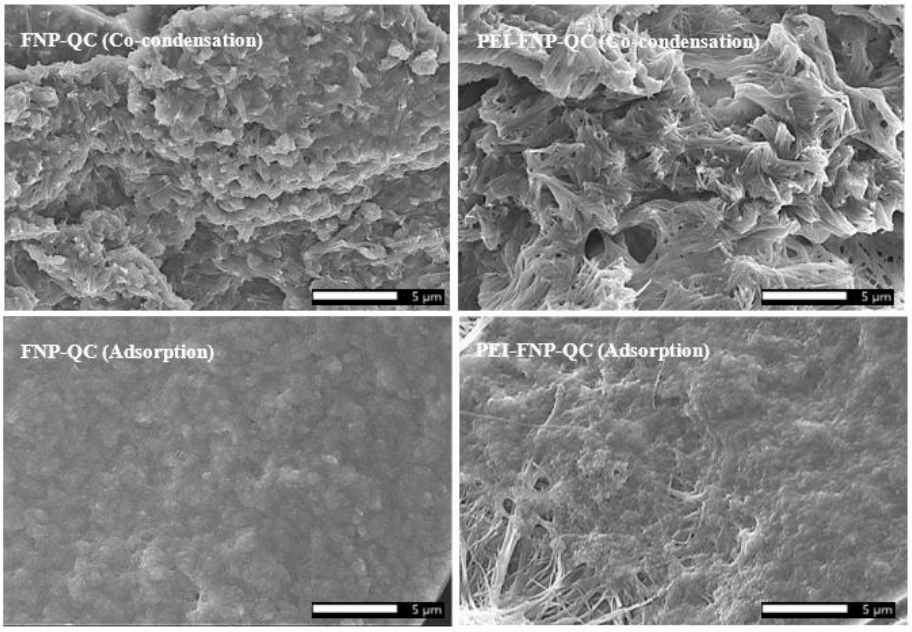

Figure 1. SEM images of FNP-QC and PEI-FNP-QC, formulated using the co-condensation and adsorption techniques.

In terms of the particle morphology, the SEM micrographs showed distinct patterns between the particles formulated by the adsorption method and the co-condensation method (**Figure 1**). As previously discussed, in the co-condensation process, the QC was precipitated on the particle surfaces, making their crystals visually appeared in the images. On the other hand, the FNP-QC and PEI-FNP-QC made by the adsorption technique demonstrated a uniform distribution of spherical particles, linked together into large arrays to create a complete nanoparticle system on which QC molecules are attached in amorphous form. This result was consistent with that of previous studies [44–46].

To elucidate the particle structures and chemical interactions, the FT-IR spectroscopy was employed (**Figure 2**). The blank FNP and PEI-FNP show the silk-II water-insoluble fibroin structure with distinguished peaks of the amide I, II, and III structures, at 1626 cm^{-1} (C=O stretch signals), 1525 cm^{-1} (N-H bending signals), and 1234 cm^{-1} (C-N stretch signals), indicating the success formulation process [38]. The PEI-FNP shows PEI characterized peak at ~2900 cm^{-1} (PEI C-H stretch), suggesting the PEI was incorporated into the FNP structure. Moreover, after encapsulating the QC, both FNP-QC and PEI-FNP-QC demonstrate QC distinctive peaks at 1666 cm^{-1} (C=O stretching vibration), 3406 or 3283 cm^{-1} (-OH stretching vibration), and at 1610, 1560, 1510 cm^{-1} (C=C stretching signals of the aromatic rings), indicating that the QC molecules were successfully loaded into the nanoparticle systems. Interestingly, the intensities of the amide I and amide III peaks of the FNP-QC and PEI-FNP-QC were reduced, compared to those of the blank counterparts, suggesting that the QC formed additional bonding with fibroin at these two locations, possibly the hydrogen bonding between oxygen and nitrogen atoms of fibroin with the hydrogen atoms of QC. Furthermore, the PEI signal of PEI-FNP-QC was also decreased, confirming the ionic interaction between negatively charged QC and positively charged PEI. Overall, these chemical interactions were in well agreement with the particles properties, as previously discussed.

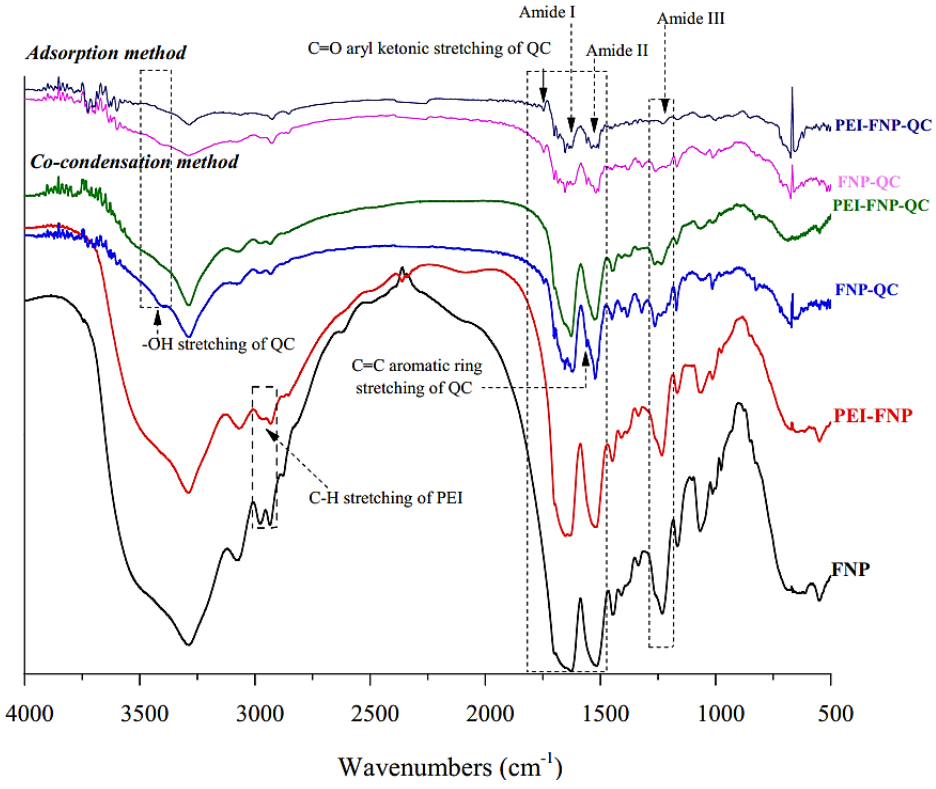

Figure 2. FT-IR spectra of the blank FNP, blank PEI-FNP, FNP-QC, and PEI-FNP-QC, formulated using the co-condensation and adsorption techniques.

For the QC water solubility test, the pure QC has very low aqueous solubility of 29.7 ± 1.2 µg/mL. Remarkably, owning to the submicron sizes, both FNP-QC and PEI-FNP-QC statistically increased the QC solubility by 2-3 times, following the order of pure QC (29.7 ± 1.2 µg/mL) < FNP-QC-Co-condensation (48.2 ± 2.3 µg/mL) < PEI-FNP-QC-Co-condensation (62.7 ± 4.7 µg/mL) < FNP-QC-Adsorption (73.1 ± 5.5 µg/mL) < PEI-FNP-QC-Adsorption (89.4 ± 6.2 µg/mL). The fact that the adsorption technique produced particles with greater QC solubility than those of the co-condensation technique was attributed to the QC polymorphs, of which the QC mainly stayed in crystalline form in co-condensation-particles and in amorphous form in adsorption-particles. This phenomenon was proven by the SEM images (**Figure 1**) and in well agreement with the previous published report [30]. Thus, the QC in co-condensation-particles encountered more difficult to be dissolved in water. Additionally, the solubility of PEI-FNP-QC was significantly higher than that of FNP-QC, which was because PEI molecules act as a surfactant, thereby helping to solubilize the QC [30].

Overall, the FNP-QC and PEI-FNP-QC were successfully formulated with appropriate properties that are beneficial for the oral administration.

3.3. Isotherm and kinetics of QC adsorption

The QC adsorption capacity over time of both the FNP and PEI-FNP is presented in **Figure 3**. The adsorption process was divided into two main stages of the rapid adsorption within 30 min, followed by a steadily-increase adsorption stage until 4 h. The PEI-FNP exhibited greater

adsorption capacities than the FNP, which reflected the complementary ionic interactions of PEI and QC, thereby enhancing the amount of drug bound on the particles.

To elucidate the mechanism of the QC adsorption process, the study described the standard isotherm (Langmuir, Freundlich, and Dubinin-Radushkevich [47] and kinetics models (pseudo-first-order and pseudo-second-order). Both FNP and PEI-FNP did not followed the Freundlich model ($R^2 < 0.9$) and followed well with the Langmuir and Dubinin-Radushkevich model, with $R^2 > 0.9$ for all formulas (**Figure 4**). This fact indicates that the QC adsorption on the particles might not be governed by multilayer process on heterogeneous surfaces. Based on the Langmuir model, we determined the highest theoretical adsorption capacity of this process was 158.7 mg/g for FNP and 192.3 mg/g for PEI-FNP. In fact, the experimental adsorption capacity of this process was ~210 mg/g for FNP and ~230 mg/g for PEI-FNP. This suggests that the QC adsorption process was mainly monolayer, with some extra interactions between the QC and fibroin/PEI molecules, possibly through the van der Waals force and hydrogen interactions [30]. Further research are necessary to elucidate this issue. Besides, with the energy of the adsorption process (E) calculated based on the Dubinin-Radushkevich model ($E = 1/\sqrt{2\beta}$) of 0.036 kJ/mol (< 8 kJ/mol) for FNP-QC and 0.033 kJ/mol (< 8 kJ/mol) for PEI-FNP-QC, it can be concluded that the nature of the adsorption process of QC onto FNP and PEI-FNP particles was mainly a physical adsorption process. This was in accordance with the FT-IR results, since most of the interactions between QC and fibroin/PEI was hydrogen bonding and ionic interactions.

Regarding the adsorption kinetics, the adsorption process of QC onto FNP and PEI-FNP particles in this study was mainly followed the pseudo-second-order kinetics model with $R^2 = 0.9998$ for FNP-QC and $R^2 = 0.9999$ for PEI-FNP-QC, instead the pseudo-first-order kinetics model with lower R^2 of 0.9744 for FNP-QC and 0.9138 for PEI-FNP-QC (**Figure 4**). This fact suggests that both FNP and PEI-FNP demonstrate rapid adsorption rates.

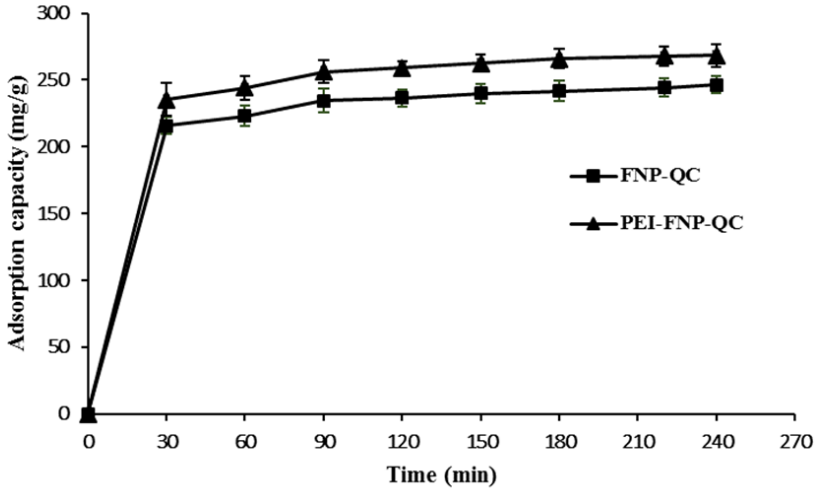

Figure 3. Adsorption capacity (mg/g) of QC on the FNP and PEI-FNP (n = 3). The adsorption process was conducted with 0.015 g of blank FNP or blank PEI-FNP, dispersed in 50 mL of QC ethanolic solution (100 µg/mL) for 4 h at 25ºC.

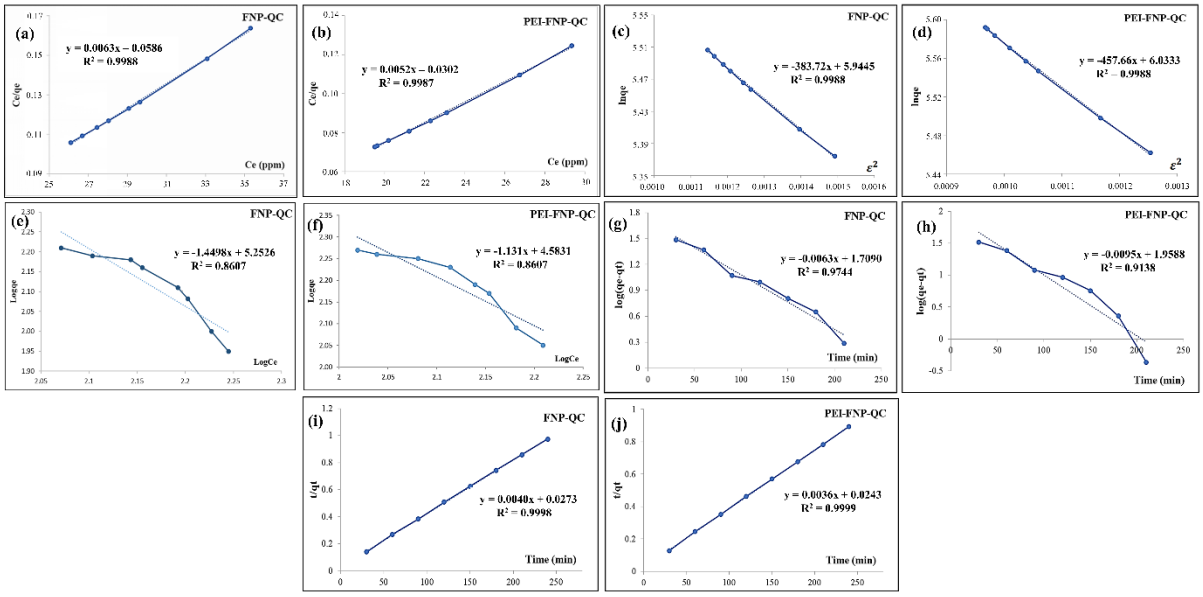

Figure 4. Adsorption isotherms (Langmuir (a, b), Dubinin-Radushkevich (c, d), and Freundlich (e, f)) and kinetics (pseudo-first-order (g, h), pseudo-second-order (i, j)) model fitting of QC on the FNP and PEI-FNP.

3.4. In-vitro QC release in simulated oral condition

To evaluate the in-vitro release processes of QC from FNP-QC and PEI-FNP-QC, the experiments were performed under conditions simulating the human gastrointestinal tract, with HCl pH 1.2 for 2 h and PBS pH 6.8 for the subsequent 6 h (**Figure 5**). In general, the QC release was limited to less than 20% QC released in the gastric juice environment for both FNP-QC and PEI-FNP-QC particles formulated by both adsorption and co-condensation technique. This result demonstrated the ability of FNP and PEI-FNP particles to protect QC in the critical gastric environment in human body. This was because both fibroin and PEI possess many different amino groups acting as basic buffer systems, thereby neutralizing the acidic micro-environment surrounding the particles [38].

During the next 6-h, under simulated intestinal conditions at pH 6.8, the QC release pattern differed between the two encapsulation methods. Roughly, the pattern followed the order of FNP-QC-Adsorption > FNP-QC-Co-condensation > PEI-FNP-QC-Adsorption > PEI-FNP-QC-Co-condensation. As such, the adsorption-particles released faster than the co-condensation-particles, and the PEI-modified particles released slower than the non-modified particles. Expectedly, as previously discussed in **section 3.2**, the co-condensation particles had most of QC molecules presented in crystalline form, with limited solubility, therefore, the QC was released slower than those of the adsorption method, which possessed QC amorphous form and higher solubility. Moreover, **section 3.3** also proves that most QC was adsorbed onto the FNP and PEI-FNP particles through a physical adsorption process, with deficient interactions and small adsorption energy. Thus, the desorption process was simple and rapid[48].

Interestingly, although the PEI-FNP-QC demonstrated higher QC solubility than those of FNP-QC, the release results of PEI-FNP-QC showed slower release efficiency than FNP-QC particles. This fact could be explained by two reasons. Firstly, as discussed in the FT-IR section, the PEI-FNP-QC form additional ionic interactions between PEI and QC, making it more difficult for the QC molecules

to be dissolved out into the buffer. Secondly, the PEI-FNP-QC particle sizes were small, with compact and tight structure [38], thus, the buffer water molecules could not freely come into contact with QC molecules in the particle cores, consequently limiting the solvating process and reducing the QC released amount.

Notably, during the whole release process, there is a slight fluctuation in the percentages of the released QC, which might be due to a part of the released QC molecules being hydrolyzed in the medium, leading to a minor decrease in total QC amounts.

In summary, the particles could protect QC from the gastric condition, while facilitating its release in the intestinal condition, which was favorable for oral administration. More importantly, one can precisely control the release rate of QC by adjusting the formulation methods (i.e., co-condensation and adsorption) and/or the PEI functionalization.

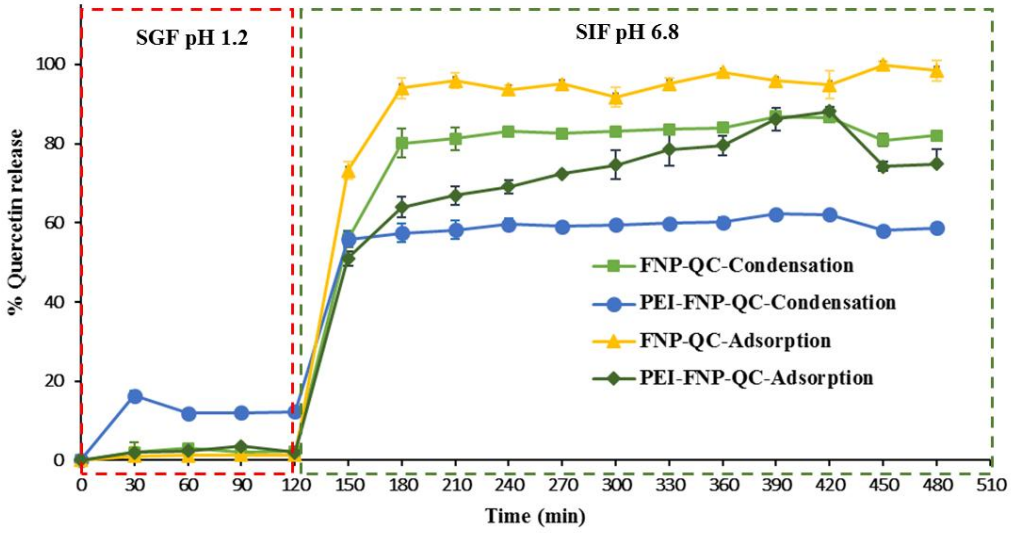

Figure 5. The release profiles of QC in simulated gastrointestinal fluid (pH 1.2 for 2 h and pH 6.8 for the next 6 h) at 37°C of FNP-QC and PEI-FNP-QC, formulated using co-condensation and adsorption techniques (n = 3).

4. CONCLUSIONS

This study prepared FNP and PEI-FNP for QC oral delivery using two techniques: adsorption and co-condensation. The formulas demonstrated appropriate physicochemical properties for oral administrations, including nano-sizes, narrow size distribution, adjustable zeta potentials, moderate QC encapsulation, smooth/rough surfaces dependent on the formulation process, observable chemical interactions, and increased drug solubility. In the simulated oral condition, the particles could protect QC from the gastric condition at pH 1.2, with less than 20% QC release, while facilitating its release in the intestinal condition. Lastly but most importantly, the fast/slow/sustain release rates of QC could be favorably controlled and adjusted by varying the formulation methods and/or PEI functionalization. The study has some limitations, including (1) the study primarily focuses on in-vitro conditions, which may not fully predict in-vivo behavior due to the complexity of biological systems, and (2) the use of PEI, while beneficial for modifying nanoparticle properties, could raise concerns about cytotoxicity and biocompatibility, which needs thorough

evaluation. Future research should focus on the in-vivo studies to assess the bioavailability, pharmacokinetics, and overall efficacy of FNP-QC and PEI-FNP-QC, the long-term stability of the particles, and the toxicity and safety evaluations. In conclusion, the FNP and PEI-FNP are potential delivery systems for oral administrations, with adjustable release properties, and could open new avenues for oral delivery of bioactive molecules.

Statements and Declarations

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Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Data availabity

The data that support the findings of this study are available from the corresponding author, Duy Toan Pham, upon reasonable request.

Author contributions

Conceptualization: P.T.M.H., D.T.P.; **methodology:** P.T.M.H., T.L.N., N.Y.N., N.T.T., M.Q.N., T.T.B.Q., D.T.P.; **investigation:** P.T.M.H., T.L.N., N.Y.N., N.T.T., M.Q.N., T.T.B.Q.; **data curation**: P.T.M.H., D.T.P.; **validation:** D.T.P.; **project administration:** D.T.P.; **resource:** D.T.P.; **writing-original draft:** P.T.M.H., D.T.P.; **writing-review and editing:** P.T.M.H., T.L.N., N.Y.N., N.T.T., M.Q.N., T.T.B.Q., D.T.P.

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جسيمات نانوية من الفيبروين معدلة ببولي إيثيلين إيمين لإطلاق خاضع للتحكم كوسيلة محتملة للتوصيل الفموي للكويرسيتين

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ملخص

تُعد المركبات متعددة الفينول فئة كبيرة من المركبات الكيميائية المستخدمة في تطبيقات طبية حيوية متنوعة. ومع ذلك، فإن هذه المركبات عرضة للتدهور، لا سيما ضمن بيئات الأس الهيدروجيني المتباينة في الجهاز الهضمي، مما يعيق استخدامها في الإعطاء الفموي. لذلك، يهدف هذا العمل إلى تطوير جسيمات نانوية من الفيبروين ((FNP)وجسيمات فيبروين معدلة ببولي إيثيلين إيمين ((PEI-FNPلحماية وتوصيل الكويرسيتين ((QC)فمويًا، باعتباره نموذجًا للمركبات متعددة الفينول. تم تحضير الجسيمات باستخدام طريقتين مختلفتين: الامتزاز والتكثيف المشترك. أظهرت الصيغتان خصائص فيزيائية–كيميائية مناسبة للإعطاء الفموي، بما في ذلك أحجام نانوية (حوالي 700 نانومتر لـ FNP-QC وحوالي 200 نانومتر لـ (PEI-FNP-QC، توزيع حجمي ضيق (مؤشر تعددية التشتت < 0.3)، إمكانات زيتا قابلة للتعديل (حوالي −20 مللي فولت لـ FNP-QC وحوالي +25 مللي فولت لـ (PEI-FNP-QC، زيادة ذوبانية الكويرسيتين في الماء بمقدار 2−3 مرات، وتفاعلات كيميائية ملحوظة (روابط هيدروجينية وتفاعلات أيونية) بين QC والفيبروين/ .PEIعلاوة على ذلك، وبناءً على عملية التحضير وتركيب الجسيمات، أظهرت الجسيمات كفاءات احتواء معتدلة للكويرسيتين (35−75%)، وأسطحًا ناعمة أو خشنة، وامتصاصًا سريعًا للدواء يتبع نماذج تشمل متساوي حرارة لانغمويير ودوبينين−رادوشكيفيتش، بالإضافة إلى حركية من الدرجة الثانية الزائفة. المثير للاهتمام أن الجسيمات، في بيئة فموية محاكية، تمكنت من حماية QC في الظروف المعدية عند pH 1.2، مع إطلاق أقل من 20%، مع استمرار إطلاقه في الأمعاء عند pH 6.8، بمعدلات يمكن التحكم بها من خلال تعديل طرق التحضير و/أو وظيفة .PEIخلاصة القول، أظهرت كل من FNP و PEI-FNP إمكانات كبيرة كنظم توصيل دوائية خاضعة للتحكم للإعطاء الفموي للمركبات متعددة الفينول.

الكلمات الدالة: الفيبروين؛ بولي إيثيلين إيمين؛ الجسيمات النانوية؛ الكويرسيتين؛ التوصيل الفموي.

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AI's Healing Touch: Transforming Healthcare from Diagnosis to Recovery

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ABSTRACT

Artificial intelligence (AI) is transforming healthcare by enhancing diagnostic accuracy, personalizing treatment, and improving patient care. This review explores the key applications of AI, including predictive analytics, machine learning, and telemedicine, which contribute to better patient outcomes, increased operational efficiency, and cost reductions. Despite these benefits, challenges such as data privacy, algorithmic bias, and regulatory compliance persist. The review emphasizes the need for healthcare professionals to receive adequate training to effectively utilize AI technologies. Addressing these challenges is essential to realizing AI's full potential in providing personalized and efficient healthcare.

Keywords: Artificial Intelligence (AI), Predictive Analytics, Telemedicine, Algorithmic Bias, Healthcare Efficiency

INTRODUCTION

Artificial Intelligence (AI) is significantly transforming the healthcare sector, revolutionizing multiple aspects of medical practice, from diagnostics to patient care and recovery [1]. By leveraging its capacity to rapidly and accurately analyze vast amounts of data, AI enables healthcare providers to make more informed decisions, enhance diagnostic precision, personalize treatment plans, and optimize patient outcomes [2]. This technological advancement is improving operational efficiency, patient outcomes, and cost management, positioning AI as a pivotal force in modern healthcare [3].

AI's impact on healthcare is particularly profound in diagnostics, where advanced algorithms can detect subtle patterns indicative of diseases such as cancer and cardiovascular conditions—often earlier than traditional methods [4]. For example, the CheXNeXt algorithm, designed to interpret chest X-rays, has demonstrated diagnostic accuracy comparable to that of human radiologists, facilitating earlier detection and intervention, which is critical for improving patient prognosis [5]. AI's ability to enhance diagnostic accuracy is reshaping the future of disease management, enabling timely and precise interventions.

Beyond diagnostics, AI is revolutionizing treatment and recovery processes. By analyzing patient data, AI enhances personalized medicine, allowing for the customization of treatment plans tailored to individual patient needs [6]. Machine learning models predict patient responses to various therapies, enabling more precise and effective interventions [7]. Additionally, AI accelerates drug discovery by identifying potential new treatments more efficiently than traditional methods, thus advancing medical research and expanding treatment options [8]. During the recovery phase, AI technologies such as remote monitoring devices and virtual health assistants provide continuous support to patients, tracking vital signs and offering personalized health information and reminders.

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This helps maintain adherence to treatment plans and improves overall care quality [9].

However, the integration of AI in healthcare is not without challenges, particularly concerning ethical considerations. One significant concern is algorithmic bias, where biases in the training data can perpetuate or even exacerbate existing healthcare disparities. Addressing this issue requires the development of bias detection tools and the use of more diverse datasets to ensure fairness and equity in AI-driven healthcare decisions [10]. Additionally, data privacy and security are critical concerns as AI systems rely on vast amounts of sensitive patient information. Ensuring compliance with regulations such as the Health Insurance Portability and Accountability Act (HIPAA) and the General Data Protection Regulation (GDPR) is essential, although these frameworks have limitations and need to evolve to address the unique challenges posed by AI technologies [11].

AI is also transforming healthcare delivery through advancements in telemedicine and virtual consultations. AI-powered systems optimize resource utilization, appointment scheduling, and service quality, making healthcare more accessible and efficient [12]. By integrating AI into telemedicine, healthcare providers can deliver more effective care while reducing operational costs [13]. The integration of AI into healthcare systems is driving significant advancements across multiple areas. From enhancing diagnostic accuracy and personalizing treatment to supporting recovery and improving healthcare delivery, AI's transformative potential is reshaping the future of medical practice [14]. As technology progresses, AI is poised to play an increasingly central role in improving patient care and advancing medical knowledge [15].

Rigorous validation is necessary to ensure reliable AI insights, and addressing algorithmic bias is crucial to prevent healthcare disparities. Integrating AI into clinical workflows requires infrastructure changes and training for healthcare professionals [16]. Despite these challenges, AI significantly enhances patient care, operational efficiency, and innovative treatments. Ongoing research, ethical oversight, and collaboration are essential to fully realize AI's potential while mitigating risks [17]. To further illustrate the impact and effectiveness of AI models in healthcare, the following section discusses real-life cases where AI has been successfully implemented. These examples highlight the transformative potential of AI in various healthcare settings, showcasing improvements in diagnosis, treatment, patient management, and operational efficiency.

Table 1: Real-Life Cases of AI Implementation in Healthcare

AI Application	Case example	Outcome	Insights	References
AI in Diagnostics	**CheXNeXt Algorithm** for Chest X-Ray Interpretation: Stanford University developed an AI model, CheXNeXt, to interpret chest X-rays and diagnose 14 different pathologies	-CheXNeXt demonstrated diagnostic accuracy comparable to that of expert radiologists. - The model enabled faster and more consistent interpretation of X-rays, reducing diagnostic errors and improving patient outcomes.	-AI models like CheXNeXt can enhance radiologists' capabilities by providing a second opinion, especially in areas with a shortage of healthcare professionals. -AI can improve diagnostic accuracy and reduce the workload on radiologists, allowing them to focus on more complex cases.	[5]

AI Application	Case example	Outcome	Insights	References
AI in Drug Discovery	**BenevolentAI** in ALS Treatment Development: Benevolent AI used machine learning algorithms to analyse biomedical data and identify a potential treatment for amyotrophic lateral sclerosis (ALS)	-The AI model successfully identified a drug candidate, which moved into clinical trials much faster than traditional methods would allow. This approach reduced the time from target identification to clinical testing, showcasing the potential to accelerate drug development.	AI can significantly reduce the timeline and costs associated with drug discovery by quickly shifting through large datasets to identify promising candidates. - Leveraging AI in drug development can increase the speed and efficiency of finding new treatments for complex diseases.	[18,19]
AI in Virtual Health Assistants	**Mayo Clinic's Chatbot** for COVID-19 Screening: During the COVID-19 pandemic, Mayo Clinic implemented an AI-driven chatbot to screen patients for symptoms and provide guidance on testing and care options.	The chatbot was highly effective in reducing the burden on healthcare facilities by managing a large volume of patient inquiries. -It helped patients take care of patients effectively, ensuring those with severe symptoms received timely care while others received remote monitoring and support.	- AI-driven virtual health assistants can play a critical role in managing public health crises by providing timely and accurate information and reducing unnecessary visits to healthcare facilities. - They improve patient engagement and satisfaction by offering convenient and accessible support.	[20]
AI in Operational Efficiency	**DeepMind's AI** for Reducing Patient Deterioration: DeepMind, a subsidiary of Alphabet Inc., developed an AI system to predict acute kidney injury (AKI) in patients, helping to reduce the incidence of AKI.	- The AI system predicted AKI up to 48 hours before it was typically diagnosed by clinicians, enabling preventative measures that reduced the number of patients requiring dialysis or intensive care	- AI can improve operational efficiency by providing early warnings for patient deterioration, allowing healthcare providers to allocate resources more effectively and improve patient management. - Such applications highlight the potential for AI to reduce complications and improve overall patient safety.	[21]

Key Takeaways from Real-Life Implementations

1. **Improved Diagnostic Accuracy and Speed**: AI models such as CheXNeXt and DeepMind's AKI prediction tool have demonstrated the ability to diagnose conditions faster and with accuracy comparable to that of human experts. This not only enhances diagnostic precision but also facilitates earlier intervention, which is crucial for patient outcomes [5].

2. **Acceleration of Drug Discovery**: AI's capacity to process and analyze vast datasets at speeds unattainable by humans has proven valuable in identifying potential drug candidates more efficiently. The case of BenevolentAI's ALS treatment candidate exemplifies how AI can significantly shorten drug development timelines, potentially bringing effective treatments to market more quickly [18,19].

3. **Effective Patient Management and Engagement**: AI-driven virtual health assistants, such as the Mayo Clinic's COVID-19 chatbot, have effectively managed patient inquiries and triaged cases, reducing the burden on healthcare facilities and improving patient engagement through accessible and personalized interactions [20].

4. **Operational Efficiency and Predictive Analytics**: AI models are enhancing operational efficiency in hospitals by predicting patient deterioration and optimizing therapy, as demonstrated by HCA Healthcare's SPOT system and DeepMind's AKI prediction tool. These systems enable earlier interventions and better resource allocation, thereby reducing mortality rates and hospital stays [21].

Challenges of AI in Healthcare: While the use of AI in healthcare offers numerous benefits, it also presents challenges that must be addressed to ensure its effective and ethical implementation [22]**.**

Table 1 : Representation of the challenges associated with AI in healthcare.

Challenge Area	Key Issues	Details	References
Data Privacy and Security	**Confidentiality** **Regulations**	Protecting sensitive patient data; vulnerability to breaches and misuse. Compliance with regulations like HIPAA (U.S.) and GDPR (Europe), adding complexity to data handling.	[23]
Data Quality and Integration	**Incomplete Data** **Data Integration**	AI models need high-quality comprehensive data; incomplete data can lead to inaccuracies. Challenges in integrating diverse data sources (e.g., EHRs, imaging systems) with varying formats.	[24]
Bias and Fairness	**Algorithmic Bias** **Equitable Access**	Potential perpetuation of biases leading to unequal treatment across demographic groups. Ensuring fair distribution of AI benefits to avoid exacerbating health disparities.	[25]
Regulatory and Ethical Issues	**Approval and Oversight** **Ethical Considerations**	Evolving regulatory landscape requires rigorous validation and oversight of AI applications. Issues like informed consent, patient autonomy, and potential misuse of AI technology.	[25,26]
Clinical Validation and Evidence	**Evidence-Based Practice** **Long-Term Impact**	Necessity for validation through clinical trials and real-world testing to ensure reliability. The need to evaluate the long-term effects of AI on patient outcomes and healthcare processes.	[26]

AI in Diagnosis

Artificial Intelligence (AI) is profoundly transforming the diagnostic landscape in healthcare, offering unprecedented precision, efficiency, and early detection capabilities [27]. The integration of AI into diagnostic processes involves leveraging advanced algorithms and machine learning techniques to analyze vast amounts of medical data, enabling more accurate and timely

identification of diseases [28].

The primary areas where AI significantly impacts diagnostics include:

- Medical imaging
- Early disease detection through predictive analytics
- Genetic analysis for personalized medicine

Medical Imaging Applications

Medical imaging is one of the most prominent areas where AI has made a substantial impact. AI-powered tools and systems are being used to increase the accuracy, speed, and consistency of image analysis in radiology, pathology, and other imaging fields [29].

Radiology:

In radiology, AI algorithms are designed to assist in interpreting medical images such as X-rays, CT scans, MRI scans, and ultrasounds. For instance, AI systems can detect abnormalities in chest X-rays that may indicate conditions such as pneumonia, tuberculosis, and lung cancer [30].

Pathology:

In pathology, AI technologies are being employed to analyze tissue samples with unprecedented speed and accuracy, aiding in the detection of cancerous cells [31]. One notable development in this field is the Augmented Reality Microscope (ARM), which integrates AI directly into traditional optical microscopes to provide real-time feedback to pathologists during examinations. By overlaying AI-generated information onto the microscope's field of view, ARM highlights areas of concern, such as potential cancerous cells, enabling faster and more accurate diagnoses. This technology allows pathologists to focus on critical regions without relying entirely on fully digital systems, making it especially useful in resource-limited environments where advanced digital pathology infrastructure may not be available [32].

Studies have demonstrated ARM's effectiveness in diagnosing breast and colorectal cancers, with performance comparable to that of trained pathologists. Its ability to identify difficult-to-detect cancer cells in real time can significantly reduce diagnostic errors, enhance decision-making speed, and improve patient outcomes [33]. Additionally, by incorporating AI directly into the microscope, the system remains accessible to a broader range of healthcare settings, offering a low-cost yet highly effective diagnostic tool.

However, while the ARM shows great potential, its adoption presents challenges such as ensuring the AI model's robustness across diverse patient populations. Pathologists must also adapt to integrating AI into their workflows, requiring both training and standardization of AI-assisted tools. Despite these challenges, ARM represents a significant advancement in AI-driven pathology, improving both the accuracy and efficiency of cancer diagnosis [34].

Ophthalmology:

AI is making significant progress in ophthalmology, particularly in the early detection and diagnosis of retinal diseases such as diabetic retinopathy, macular degeneration, and glaucoma. By analyzing retinal scans with high precision, AI systems can detect these conditions earlier than traditional methods, enabling timely intervention and preventing vision loss [35]. AI algorithms, such as those developed by Google's DeepMind, have demonstrated the ability to diagnose eye conditions with accuracy comparable to that of human specialists, significantly improving patient outcomes through early diagnosis and treatment.

Beyond diagnosing retinal diseases, recent research has explored the use of AI algorithms to predict systemic diseases from retinal images. As discussed by Khan et al. (2023), AI systems are now capable of identifying signs of cardiovascular diseases, diabetes, and even kidney disease by analyzing subtle patterns in retinal scans. This cross-domain application of AI is transforming retinal imaging from a tool used solely for ophthalmic diagnoses to one that provides valuable insights into a patient's overall systemic health. Such predictive capabilities offer a non-invasive and efficient way to screen for systemic conditions, potentially leading to earlier interventions and improved disease management [36].

Early Disease Detection with Predictive Analytics

Predictive analytics is another domain where AI is rapidly evolving in healthcare diagnostics [37]. AI systems can analyze patient data such as electronic health records (EHRs), lifestyle information, and genetic data to predict the risk of certain illnesses. This capability enables early intervention and supports preventive care [38].

Cardiovascular Diseases:

AI algorithms are increasingly being used to predict cardiovascular events such as heart attacks and strokes [39]. For instance, the Framingham Heart Study has employed machine learning techniques to develop predictive models that assess the risk of cardiovascular diseases based on factors such as age, cholesterol levels, and blood pressure [40]. By identifying patterns and risk factors in large datasets that might be overlooked by traditional statistical methods, AI enables more accurate risk assessments and supports preventive care measures [41].

Diabetes:

In diabetes care, AI-driven predictive analytics can forecast the onset of type 2 diabetes by analyzing patient data, including glucose levels, family history, and lifestyle factors [42]. Early prediction enables timely lifestyle modifications and interventions that can delay or even prevent disease onset.

Cancer:

AI is also playing an important role in the early detection of cancer. Predictive analytics can identify individuals at high risk of developing cancers such as breast, colorectal, and prostate cancer [43]. By analyzing genetic markers, family history, and lifestyle factors, AI systems can recommend early screening and monitoring, leading to earlier detection and improved outcomes [44].

Moreover, AI is increasingly contributing to post-cancer care by providing personalized psychological support and cognitive rehabilitation to survivors. AI-driven platforms analyze patient data, including medical histories and psychological profiles, to offer tailored mental health interventions. These systems, often powered by virtual assistants and chatbots, guide patients through therapies such as cognitive behavioral therapy (CBT) and mindfulness exercises, offering continuous support. By addressing psychological challenges such as anxiety, depression, and cognitive decline, AI enhances the holistic recovery process and improves the quality of life for cancer survivors [45].

Genetic Analysis for Personalized Medicine

Genetic analysis is a rapidly advancing field in which AI plays a critical role. By analyzing genetic data, AI helps uncover the genetic foundations of diseases and supports the development of personalized treatment plans tailored to an individual's genetic profile [46].

Genomic Sequencing:

AI algorithms are employed to analyze and interpret the vast amounts of data generated by genomic sequencing. Machine learning models can identify genetic mutations and variants associated with specific diseases [47]. This is particularly valuable in oncology, where identifying genetic mutations can inform targeted therapies.

Pharmacogenomics:

Pharmacogenomics, which examines how genes influence an individual's response to medications, is another area where AI is making significant advancements. By analyzing genetic data, AI can predict how different patients will respond to medications, enabling personalized drug prescriptions that minimize adverse effects and maximize efficacy [48]. For example, the AI platform developed by Tempus analyzes both clinical and molecular data to provide insights into optimal treatment options for cancer patients based on their genetic profiles [49].

Rare Diseases:

AI is also being used to diagnose rare genetic disorders. By comparing a patient's genetic data with extensive databases of known genetic conditions, AI can identify potential rare diseases that might be missed by traditional diagnostic approaches [50].

Table 2: Outlines how AI works in diagnosis across different applications.

Application	AI Mechanism	How AI Works	References
Medical Imaging	Deep Learning, Convolutional Neural Networks (CNNs)	AI analyses medical images, including X-rays, CT scans, and MRIs, to identify irregularities and diagnose diseases.	[29]
Pathology	Statistical Learning, Image Recognition	AI scans and interprets tissue samples to identify cancerous cells and other abnormalities. E.g.: Augmented Reality Microscope (ARM)	[31,32]
Ophthalmology	Neural Networks, Image Analysis	AI analyses retinal scans to detect eye diseases like diabetic retinopathy and macular degeneration.	[36]
Early Disease Detection	Predictive Analytics, Machine Learning Algorithms	AI predicts the likelihood of diseases by analysing patient data, including EHRs, lifestyle, and genetic information.	[37,38]
Genetic Analysis	Genomic Sequencing, Machine Learning	AI interprets genetic data to identify mutations and variants linked to diseases	[46]
Pharmacogenomics	Data Analytics, Machine Learning	AI predicts patient responses to medications based on genetic data, personalizing drug prescriptions.	[48,49]

AI in Treatment

Artificial Intelligence (AI) is revolutionizing healthcare by enabling more personalized, efficient, and effective medical interventions [51]. From creating customized treatment plans to accelerating drug discovery and enhancing surgical precision, AI is transforming how healthcare providers deliver care [52].

Personalized Treatment Plans Using Data Analysis:

Personalized treatment plans are a cornerstone of precision medicine, and AI is instrumental in tailoring these plans to individual patients' needs. By examining large volumes of data—including genetic information, lifestyle factors, and clinical history—AI can develop highly customized treatment strategies [53].

Integrating and Analysing Data:

AI algorithms aggregate information from multiple sources, such as electronic health records (EHRs) and genomic databases. Machine learning models analyze this data to uncover patterns and correlations that guide treatment decisions [54].

Predictive Modelling:

AI uses predictive modeling to forecast how patients will respond to different treatments. These models are trained on historical data, including treatment outcomes,

side effects, and patient demographics [55]. By predicting the efficacy and potential adverse reactions of treatments, AI helps clinicians select the best therapeutic options for each patient. For instance, IBM Watson for Oncology employs machine learning to recommend personalized cancer treatment strategies tailored to a patient's genetic profile and current medical research [56].

Dynamic Treatment Adjustments:

AI enables real-time monitoring and adjustment of treatment plans. Wearable devices and remote monitoring tools collect continuous health data, which AI analyzes to detect changes in a patient's condition. This allows for dynamic treatment adjustments, ensuring that interventions remain effective over time [57].

Case Studies:

In cardiology, AI algorithms have been utilized to tailor treatments for patients with atrial fibrillation by predicting which antiarrhythmic drugs are most likely to prevent recurrence [58]. AI models, particularly those using machine learning algorithms like neural networks, process large datasets—including heart rhythm data, genetic markers, and clinical outcomes—to determine which patients should receive specific antiarrhythmic drugs such as amiodarone or flecainide [59].

In oncology, the **Tempus** platform integrates AI to process molecular profiles and clinical information, developing treatment strategies for cancer. Using support vector machines (SVMs) and deep learning models trained on large genomic datasets, patient data, and drug efficacy information, Tempus compares a patient's tumor molecular profile—including genetic mutations like *BRCA1/BRCA2*, *EGFR*, or *KRAS*—to recommend the most effective therapy [60].

AI-Driven Drug Discovery and Development:

Drug discovery and development are traditionally lengthy, costly, and have high failure rates. AI significantly accelerates this process by identifying potential drug candidates more efficiently and predicting their likelihood of success in clinical trials [61].

Identifying Drug Candidates:

AI algorithms screen vast chemical libraries to identify molecules with potential therapeutic effects. Machine learning models analyze chemical structures and predict their biological activity, dramatically reducing the time required to identify promising candidates [62]. For example, Atomwise uses AI to predict the binding affinity of small molecules to target proteins, streamlining the initial phase of drug discovery.

Predicting Drug Efficacy and Safety:

AI models predict the efficacy and safety of drug candidates by analyzing preclinical and clinical data. These models simulate how drugs interact with biological pathways and predict potential side effects [63]. This prioritizes the most promising candidates for clinical trials, improving overall success rates.

Optimizing Drug Design:

AI assists in optimizing chemical structures to enhance drug efficacy and reduce toxicity. By iteratively analyzing the relationship between a drug's structure and its biological activity, AI suggests modifications that improve performance [64].

AI in Patient Care

Artificial Intelligence is transforming patient care by enhancing engagement, facilitating remote monitoring, improving telemedicine, and enabling predictive analytics for better health management [65].

Virtual Health Assistants for Patient Engagement

Virtual health assistants (VHAs) are AI-driven platforms designed to interact with patients through digital channels such as chatbots, mobile apps, and voice assistants. They handle tasks ranging from answering health-related questions to reminding patients about medication schedules [66].

Personalized Patient Interaction :

VHAs tailor interactions based on medical history, preferences, and current health status [67]. For example, a VHA can remind a diabetic patient to check glucose levels, provide dietary advice, and suggest physical activities.

24/7 Availability:

Unlike human providers, VHAs offer continuous support, ensuring patients have access to information at all times, which reduces anxiety and improves satisfaction [68].

Health Education and Support:

Virtual Health Assistants (VHAs) can educate patients about their conditions and treatment plans. For instance, a VHA can explain the side effects of medications, offer tips for managing symptoms, and provide information on lifestyle changes that can improve health outcomes. This educational role is crucial in encouraging patients to take an active role in their treatment [69].

Reducing Administrative Burden:

By automating routine tasks such as appointment scheduling, prescription refills, and responses to common health questions, VHAs reduce the administrative burden on healthcare providers. This allows healthcare professionals to focus more on direct patient care and complex medical concerns [70].

Remote Monitoring and Telemedicine's Impact on Care

Remote monitoring and telemedicine have transformed patient care by enabling continuous health tracking and providing access to medical consultations without the need for in-person visits. AI enhances these technologies, making them more effective and user-friendly [71].

Continuous Health Monitoring:

AI-powered devices such as smartwatches and wearable sensors collect real-time data on vital signs and activity levels. AI algorithms analyze this data to detect anomalies, such as signs of atrial fibrillation, and alert healthcare providers [72].

Early Detection and Intervention:

AI continuously monitors health data to detect early indicators of disease or exacerbations of chronic illnesses, allowing for timely interventions. For example, AI can predict asthma attacks by tracking environmental factors and patient symptoms [73].

Telemedicine Integration:

AI supports telemedicine by offering diagnostic insights and decision-making tools during virtual consultations, enhancing the accuracy and efficiency of care [74].

Patient Convenience and Access:

Remote monitoring and telemedicine reduce the need for in-person visits, improving access to healthcare, particularly for patients in rural or underserved regions [75].

Reducing Healthcare Costs: By enabling early detection and reducing hospital admissions, these technologies lower healthcare costs and support more cost-effective care through virtual consultations [76].

Predictive Analytics for Managing Patient Care

Predictive analytics leverages AI to analyze historical and real-time data to forecast future health events, enabling proactive and preventive care management. This approach is transforming how healthcare providers manage patient care [77].

Risk Stratification:

AI-driven predictive models can categorize patients according to their risk of developing specific conditions or experiencing adverse health events [78].

Personalized Care Plans:

By forecasting how patients may respond to different treatments, AI helps healthcare providers develop personalized care plans that account for individual risk factors, genetic profiles, and lifestyle habits. For example, AI can predict which patients are most likely to benefit from specific medications based on their genetic makeup and clinical history [79].

Chronic Disease Management:

Predictive analytics is particularly valuable for managing chronic diseases. AI models can forecast disease progression and suggest interventions to prevent complications. For example, predictive analytics can predict blood glucose levels in diabetic patients, enabling personalized insulin therapy and dietary recommendations [80].

Improving Patient Outcomes:

Predictive analytics supports early intervention and personalized care, leading to improved disease management and enhanced quality of life [81].

Operational Efficiency:

Predictive analytics helps healthcare providers optimize resource allocation by forecasting patient admission rates and adjusting staffing levels to meet demand [82].

Table 3: Outlines how AI works in patient care.

Heading	Description	Examples
Virtual Health Assistants for Patient Engagement	**Personalized Patient Interaction:** Tailors interactions based on individual patient data (medical history, preferences, current health status) [67]. **24/7 Availability:** Provides immediate responses to patient inquiries anytime [68]. **Health Education and Support:** Educates patients about conditions, treatment plans, and lifestyle changes [69]. **Reducing Administrative Burden:** Automates routine tasks like appointment scheduling and prescription refills [70].	Ada Health: Guides patients through a set of questions regarding their symptoms to offer potential causes and next steps. Buoy Health: Diagnoses patients based on symptoms and suggests appropriate actions
Remote Monitoring and Telemedicine's Impact on Care	**Continuous Health Monitoring:** AI-powered devices gather real-time information on vital signs and health metrics [72]. **Early Detection and Intervention:** Identifies early signs of diseases or chronic condition exacerbations for timely intervention [73]. **Telemedicine Integration:** Provides diagnostic support and decision-making tools during virtual consultations [74]. **Patient Convenience and Access:** Decreases the necessity for in-person visits, improving access to healthcare [75,76]. **Reducing Healthcare Costs:** Lowers costs by enabling early detection and	Livongo: Provides personalized insights and recommendations for managing chronic conditions. Amwell: Integrates AI to assist doctors during virtual visits.

Heading	Description	Examples
	reducing hospital admissions [77].	
Predictive Analytics for Managing Patient Care	**Risk Stratification:** Identifies patients who are at high risk of developing conditions or encountering adverse events [78]. **Personalized Care Plans:** Predicts patient responses to treatments for personalized care plans [79]. **Chronic Disease Management:** Forecasts disease progression and suggests interventions [80]. **Improving Patient Outcomes:** Enables early intervention and personalized care for better outcomes [81]. **Operational Efficiency:** Optimizes resource allocation and operational efficiency [82].	Sepsis Watch: Identifies patients at risk of sepsis for early intervention. Cleveland. Clinic's Predictive Model: Identifies patients at high risk of cardiac arrest for preventive measures.

Future Prospects and Innovations

The future of AI in healthcare appears highly promising, with numerous emerging technologies and trends expected to further transform the field. Equally important is the education and training of healthcare professionals, ensuring they are well-equipped to effectively utilize and integrate these advanced technologies into patient care [83].

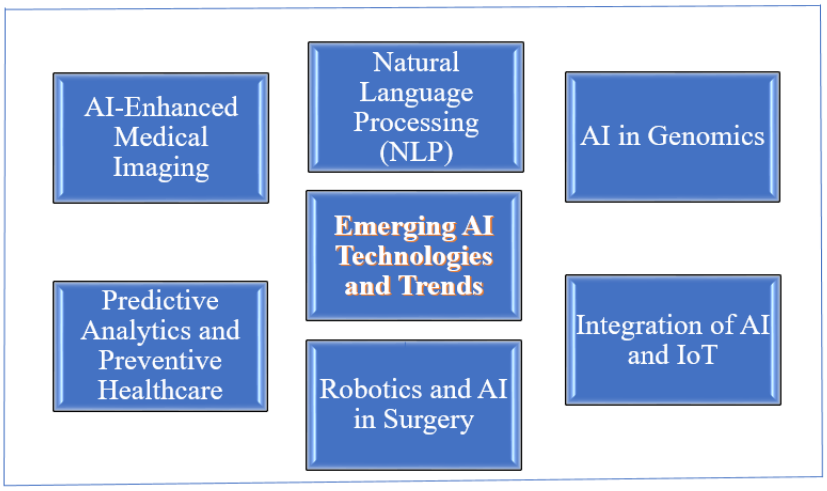

Figure 1. Emerging AI Technologies and Trends.

1. AI-Enhanced Medical Imaging:

AI algorithms are becoming increasingly sophisticated in analyzing medical images. Future advancements will likely enable AI not only to identify abnormalities in X-rays, MRIs, and CT scans but also to provide comprehensive diagnostic insights and even suggest treatment options [84]. For instance, AI could detect early indicators of diseases such as cancer—potentially before they are noticeable by human radiologists—allowing for earlier and more effective interventions.

2. Natural Language Processing (NLP):

NLP technologies are advancing rapidly, enabling AI systems to better understand and interact with human language. This has significant implications for patient care, including more accurate transcription of medical records, extraction of actionable insights from clinical notes, and even direct patient interaction through chatbots [85]. Enhanced NLP capabilities could streamline documentation processes, improve data accuracy, and support more efficient patient data management.

3. AI in Genomics:

The integration of AI with genomics represents another promising trend. AI can process vast amounts of genetic data to uncover patterns and correlations that would be challenging for humans to detect [86]. This enables the development of personalized medicine, where treatments are customized according to an individual's genetic profile. AI-driven genomics can identify genetic predispositions to diseases and help develop targeted therapies.

4. Predictive Analytics and Preventive Healthcare:

Predictive analytics powered by AI is poised for significant growth, enabling healthcare providers to foresee and prevent health issues before they become critical [87]. By analyzing data from multiple sources—including wearable devices, electronic health records, and lifestyle information—AI can predict disease likelihood and recommend preventive measures [88]. This shift toward preventive healthcare can improve patient outcomes while reducing healthcare costs.

5. Robotics and AI in Surgery:

Robotic surgery enhanced by AI will continue to evolve, making procedures less invasive and more precise [89]. AI can assist in preoperative planning, provide real-time decision support during surgery, and optimize postoperative recovery management. As this technology advances, it is expected to expand to a wider range of surgical specialties, improving patient recovery times and reducing complications.

6. Integration of AI and IoT:

Integrating AI with the Internet of Things (IoT) is creating smarter healthcare ecosystems. Connected devices continuously monitor patients' health and transmit real-time data to AI systems, which analyze the information to generate insights and alerts [90]. This integration enables proactive remote patient monitoring and chronic disease management, leading to more personalized and responsive healthcare delivery.

CONCLUSION

The application of artificial intelligence in healthcare offers a promising pathway to improve the quality, efficiency, and effectiveness of medical services. AI facilitates early diagnosis, supports a range of treatment options, enhances patient outcomes, and optimizes healthcare operations. However, critical challenges—including data privacy, algorithmic bias, and equitable access to AI technologies—must be addressed. AI systems must comply with regulatory requirements such as HIPAA and GDPR to safeguard patient data while also preventing algorithmic biases to ensure equitable healthcare delivery across all populations.

Future development of AI in healthcare should focus on continuous research, ethical oversight, and real-world validation to maximize its positive impact. Equally important is the training of healthcare professionals to effectively integrate AI into clinical workflows. By addressing these core areas, the healthcare sector can fully leverage AI to deliver more personalized, efficient, and equitable care.

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لمسة الذكاء الاصطناعي العلاجية: تحويل الرعاية الصحية من التشخيص إلى التعافي. مراجعة شاملة لتطبيقات الذكاء الاصطناعي في أنظمة الرعاية الصحية الحديثة

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ملخص

يتزايد اعتماد الذكاء الاصطناعي في مجال الرعاية الصحية، وقد بدأ يُحدث تأثيرًا ملموسًا على جوانب مُختلفة من هذا القطاع، بدءًا من التشخيص واستراتيجيات العلاج وصولًا إلى رعاية المرضى. نناقش هذه التطبيقات في هذه المراجعة باستخدام الذكاء الاصطناعي، بما في ذلك التحليلات التنبؤية وتقنيات التعلم الآلي، بالإضافة إلى الطب عن بُعد والمراقبة عن بُعد المُعتمدة على الذكاء الاصطناعي. تُوفر هذه التطورات فوائد كبيرة لرعاية المرضى، وتُحسّن الكفاءة التشغيلية، وتُخفّض التكاليف. إلا أن هناك تحديات حقيقية في مجالات مثل خصوصية البيانات، والتحيز الخوارزمي، والامتثال. تُضيف هذه المراجعة إلى الدراسات السابقة أن مُختصي الرعاية الصحية بحاجة إلى التدريب قبل أن يتمكنوا من الاستفادة المثلى من تقنيات الذكاء الاصطناعي. كلما زادت تعقيداتها، زاد تأثيرها على الرعاية الصحية، مُحسّنةً رعاية المرضى بطريقة تُناسب مرضانا على حدة، دون النظر إلى كل مرض من منظور خارجي وداخلي فقط.

الكلمات الدالة: الذكاء الاصطناعي، التحليلات التنبؤية، التعلم الآلي، الطب عن بعد، رعاية المرضى، التحيز الخوارزمي.

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تاريخ استلام البحث 2024/08/07 وتاريخ قبوله للنشر 2024/09/21.

Swietenia mahagoni Leaves Ethanolic Extract: *In vitro* anti-Oxidant Activity, Active Compound Identification and *in silico* Prediction as AKT-1 and MDM2 Protein Inhibitor

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ABSTRACT

The strong correlation between traditional practices and the pharmacological properties of these plants supports their continued use in treating various health conditions. This study evaluated and predicted the active compound in the ethanolic extract of *Swietenia mahagoni* leaves and their potency for inhibiting cancer cell growth. The analysis included measuring DPPH free radical inhibition, total phenolic and flavonoid content, drug-likeness evaluation, and molecular docking studies. Findings suggest that the ethanolic extract of *S. mahagoni* leaves ethanolic extract exhibits antioxidant properties due to its content of phenolic and flavonoid compounds such as Quercitrin, (+)-ar-Turmerone, and Hyperoside, which also meet Lipinski's criteria. Additionally, these compounds might act as inhibitors of MDM2 or AKT-1, potentially blocking MDM2 and AKT-1 and inducing apoptosis in cancer cells. Further research should be conducted in vitro to validate the activity of the studied compounds.

Keyword: Anticancer, Insilico, Plant-based medicine, Secondary metabolite, *Swietenia mahagoni*

1. INTRODUCTION

The incidence of cancer is increasing rapidly, fueling the search for new treatments. Although progress in cancer research is accelerating, only a few drugs make it through clinical trials successfully [1,2]. Cancer cells are categorized according to their origin or the genetic mutations involved in their growth [3,4]. Effective treatment usually requires a combination of drugs designed to target the unique characteristics of the cancer cells. As a result, there is growing interest in developing novel anticancer therapies from natural sources [5–7].

Traditional healthcare practices include a diverse array of methods, prominently featuring plant-based remedies used alone or in combination to treat diseases in humans and animals globally [8]. These practices span various traditional systems such as ancient Korean, Malay, Chinese and Indian and African medicines [9]. Indonesian folk medicine is noted for its distinctive diagnostic and treatment methods, leveraging the country's rich biodiversity and diverse indigenous cultures. For centuries, Indonesian communities have relied heavily on plant-based remedies, with about 85% of their healthcare formulations derived from plants [10].

The popularity of herbal remedies is due to their cultural acceptance, cost-effectiveness, perceived efficacy, and minimal side effects [11,12]. Recent research has focused on analyzing medicinal plants recommended by traditional practitioners to identify bioactive compounds and enhance drug discovery. The strong correlation between traditional practices and the pharmacological

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properties of these plants supports their continued use in treating various ailments [13,14]. Additionally, conducting drug-likeness analysis is essential for discovering high-quality drug candidates from herbal medicine, as it helps avoid unnecessary biological testing and clinical trial expenses [15,16].

Swietenia mahagoni is utilized as a medicinal plant in various regions, including India (within the Ayurvedic system), several African countries, and in Indonesia and Malaysia. Traditionally, it is used to treat malaria, hypertension, diabetes, and diarrhoea, and serves as an antipyretic, bitter tonic, and astringent[17]. The pharmacological properties of *S. mahagoni* include antimicrobial, anti-inflammatory, hepatoprotective, neuropharmacological, anti-diabetic, immunomodulatory, and anticancer activities [18]. However, the anticancer mechanism of this plant has yet to be widely studied. Thus, this study evaluated and predicted the active compound that is contained in *S. Mahagoni* ethanolic extract and their potency for inhibiting cancer cell growth.

2. MATERIAL AND METHODS

2.1 *Swietenia mahagoni* leaves extraction process

Powder dried leaves of *S. mahagoni* was obtained from Materia Medica Batu, Batu, Indonesia. Extraction processes were conducted at Pharmacology Laboratorium, Universitas Brawijaya, Malang, Indonesia using ethanol as the solvent. Powder leaves were macerated in ethanol with ration 1:10 w/v (g/mL) for 24 hours. The macerated was filtered using filter pape. The extract was dried using rotary evapotaror in temperature 80°C.

2.2 Antioxidant analysis from the extract

The antioxidant activity which produced by extracts was analysis with 2,2-diphenyl-1-picrylhydrazyl (DPPH) [1] free radical. In this study, a freshly prepared DPPH solution (0.2 mM) in 100% ethanol was employed. *S. mahagoni* extracts and the equivalent amount (100 µL) of the DPPH solution were combined in a microplate (Costar 96-well plate). The reaction mixture was let remain in the dark at room temperature (25 °C) for 30 minutes. For the control, the same amount (100 µL) of ethanol and DPPH solution were combined. Using a microplate spectrophotometer (SPECTROstar Nano - BMG LABTECH, Germany), the absorbance value of the reaction mixtures was determined at 517 nm. Using Equation 1, the DPPH radical scavenging activity was determined. The IC50 value was calculated by interpolating the fifty values to the linear regression equation (R2 > 0.99) derived from the inhibition curve.

$$\text{DPPH inhibition (\%)} = \left(\frac{\text{AB} - \text{AS}}{\text{AB}}\right) \times 100 \qquad \text{Equation (1)}$$

where, AB is absorbance of blank; AS is absorbance of the sample

2.3 Total flavonoids assay

The colorimetric evaluate with aluminium chloride was carried out to determine the total flavonoids content [1]. The standard of reference was quercetin with concentration of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 mg/mL. The 0.001 g/mL of *S. mahagoni* ethanolic extract was used for sample. The 150 µL of 96% ethanol solution was added after about 50 µL of *S. mahagoni* ethanolic extract or standard was added to 10 µL of aluminium chloride (10% w/v). Next, 10 µL of 1M concentration sodium acetate (Sigma) was added to the solution. For forty minutes, the solution was kept out of the light and incubated at room temperature. At $\lambda = 405$ nm, the absorbance was measured with a BioTek ELx808 ELISA reader. The total flavonoid concentration was reported as mg QE/g w.b. of dry extract, which is comparable to quercetin, according to the quercetin standard curve equation.

2.4 Total phenolic content analysis

The Folin-Ciocalteau procedure was used to analyze the total phenolic content [19]. The standard of reference was gallic acid with concentration of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 mg/mL. The 0.001 g/mL of *S. mahagoni* ethanolic extract was used for sample In a 96-well microplate, 30 µL of 1.0 N Folin-Ciocalteu reagent was

combined with 60 µL of samples (standard and *S. mahagoni* ethanolic extract), and the mixture was incubated for 5 minutes. Next, 150 µL of a 20% sodium carbonate solution was added, and the mixture was allowed to sit at room temperature for 40 minutes in a dark area. After 8 minutes of centrifugation (1600×g), the absorbance of the the suspension was measured using a Microplate Reader at 730 nm. Gallic acid (mg GAE/g w.b.) was used in a standard curve to determine the samples' total phenolic content.

2.5 Screening and identification of bioactive compounds in phytochemicals

The phytochemical content in seagrass extracts was analyzed using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) with a Q-exactive model from Thermo Fisher Scientific. 1400 µL of modified solvents (water, 50% ethanol, or 100% ethanol) were used to dissolve 100 µL of *S. mahagoni* extract. Samples were introduced into the LC-HRMS device after being filtered using a 0.22 m RC minisart. A total of ten milliliter samples will be automatically processed using a hypersil gold aQ 50 × 1 mm × 1.9 column at positive polarity conditions, with a flow rate of forty liters per minute and an oven column temperature of thirty degrees Celsius. The elution gradient will be as follows: 5% B for two minutes, 60%-95% B for fifteen to twenty-two minutes, and then 5% B for thirty minutes. Utilizing the Compound Discoverer 3.1 program, which is based on the mzcloud, the chromatogram data produced by the injection procedure will be examined to identify the compounds [19]

2.6 ADME and Drug-Likeness Analysis

The online web server pkCSM (http://biosig.unimelb.edu.au/pkcsm/) can be utilized to predict ADME (absorption, distribution, metabolism, and excretion), while the web server at (https://tox-new.charite.de/protox_II/) is available for predicting toxicity and evaluating the Lipinski Rule.

2.7 Ligand and protein structures preparation

The ligands used for docking analysis were Quercitrin, (+)-ar-Turmerone, Hyperoside, an imidazole (an MDM2 inhibitor) and AZD5363 (an AKT-1 inhibitor). The 3D structures of three ligands (Quercitrin, (+)-ar-Turmerone, and Hyperoside) were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The 3D structure for imidazole was obtained from native ligand in MDM2 protein (PDB ID, 4OQ3) from PDB database. The 3D structure for AZD5363 was obtained from native ligand in AKT-1 protein (PDB ID, 4GV1) from PDB database. The proteins were cleaned using BIOVIA Discovery Studio software.

2.8 Ligand docking studies

AutoDock Vina integrated in PyRx 0.8 was used to analyze interactions between ligands and proteins. In order to assess binding affinities and elucidate molecular pathways, the docking approach was applied. In order to execute docking, ligands were made into flexible molecules and receptors into stiff molecules inside the active site. By using BIOVIA Discovery Studio, docking and binding interaction results were examined.

3. RESULT

3.1 Antioxidant activity of *S. mahagoni* ethanolic extract

The antioxidant activity some compounds could be determined by their ability to inhibit free radical, such DPPH free radical. *S. mahagoni* ethanolic extract showed their ability to inhibit DPPH free radical manner by concentration (Table 1). Interestingly, about 25 ppm of *S. mahagoni* ethanolic extract, DPPH free radical was inhibited around 50%. This early finding has positive value for *S. mahagoni* ethanolic extract for their bioactivity.

Table 1. Antioxidant activity analysis from the *S. mahagoni* ethanolic extract

Concentration (ppm)	DPPH inhibition (%)
0.39	2.32 ± 0.11
0.78	4.30 ± 1.25
1.56	7.03 ± 0.35
3.13	11.12 ± 0.62
6.25	18.42 ± 0.77
12.50	32.47 ± 0.85
25	50.14 ± 0.11
50	84.99 ± 1.30

3.2 Total phenolic and Total flavonoid of *S. mahagoni* ethanolic extract

Phenolic and Flavonoid are secondary metabolites which support pharmaceutical effect in plant, as well as *S. mahagoni* ethanolic extract. Total flavonoid and phenolic content could be imaging the pharmaceutical effect the plant. *S. mahagoni* ethanolic extract measured contain TFC 458.91 ± 21.60 (mgQE/g w.b) and TPC 299.90 ± 14.28 (mgGAE/g w.b) (Table 2)

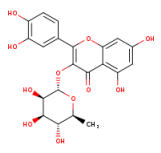
Table 2. Total phenolic and flavonoid contents of *S. mahagoni* ethanolic extract (SME)

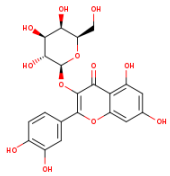
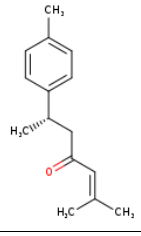
Sample	TFC (mgQE/g w.b)	TPC (mgGAE/g w.b)
SME1	453.38	298.72
SME2	435.66	283.02
SME3	487.69	317.95
Mean	458.91 ± 21.60	299.90 ± 14.28

3.3 Compound identification of *S. mahagoni* ethanolic extract using LC-HRSM analysis

The identification of active compound in *S. mahagoni* ethanolic extract was continued with LC-HRMS analysis. Around 40 compound was found in in *S. mahagoni* ethanolic extract. Three active compound was selected for future analysis, there are 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-{[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan--2-yl]oxy}-4H-chromen-4-one (Quercitrin), (+)-ar-Turmerone, Hyperoside, more information as shown in Table 3. Future analysis with these compounds including druglikeness analysis and molecular interactions prediction.

Table 3. Characteristics of active compound from *S. mahagoni* ethanolic extract

Name	Structure	Formula	Calc. MW	RT [min]	Area (Max.)	mzCloud Best Match
Hyperoside		C21 H20 O12	464.09	7.16	5.42E+07	99.3

Name	Structure	Formula	Calc. MW	RT [min]	Area (Max.)	mzCloud Best Match
Quercitrin		C21 H20 O11	448.10	7.84	2.18E+07	99.1
(+)-ar-Turmerone		C15 H20 O	216.15	17.48	1.60E+07	98.1

3.4 Drug likeness analysis based on Lipinsky's role and ADME analysis

A druglikeness analysis was conducted based on Lipinsky's role which supported with ADME analysis. Table 4 presents a drug-likeness analysis that is categorized into four parameters: molecular mass, hydrogen bond donors, hydrogen bond acceptors, and high lipophilicity. Overall, two of four criterias were effective in Quercitrin, (+)-ar-Turmerone, and Hyperoside. Additionally, pharmacokinetic information on the toxicity, excretion, metabolism, distribution, and absorption of hyperoside, quercitrin, and (+)-ar-Turmerone was assessed in relation to the ADME study (Table 5).

Table 4. Lispinsky's role of Quercitrin, (+)-ar-Turmerone, and Hyperoside

Lipinsky's role	Hyperoside	Quercitrin	(+)-ar-Turmerone
Molecular mass	464.38	448.10	216.32
High lipophilicity	0.54	0.49	4.02
Hydrogen bond donors	8	7	0
Hydrogen bond acceptors	12	11	1

Table 5. ADME prediction of Quercitrin, (+)-ar-Turmerone, and Hyperoside

Properties	Parameters		Ligands		
			Hyperoside	Quercitrin	(+)-ar-Turmerone
Absorption	Water Solubility (log mol/L)		-2.92	-2.90	-4.45
	Intestinal Absorption (%)		48.00	52.71	94.49
Distribution	Volume Distribution (VDss) (log L/Kg)		1.85	1.52	0.62
Metabolism	Inhibitor of	CYP1A2	-	-	-
		CYP2C19	-	-	-
		CYP2C9	-	-	-
		CYP2D6	-	-	-
		CYP3A4	-	-	-
Excretion	Total Clearance (mL/min/kg)		0.39	0.36	0.29

3.5 Molecular docking analysis

The docking results for MDM2 and AKT-1 with the three compounds found in *S. mahagoni* ethanolic extract—Quercitrin, (+)-ar-Turmerone, and Hyperoside showed binding affinities of −7.5, −6.9, and −7.3 kcal/mol for MDM2, respectively, compared to a known inhibitor, imidazole, which has a binding affinity of −9.6 kcal/mol. For AKT-1, Quercitrin, (+)-ar-Turmerone, and Hyperoside exhibited binding affinities of −7.8, −6.5, and −8.0 kcal/mol, respectively, in contrast to the known inhibitor AZD, which has a binding affinity of −9.1 kcal/mol. Furthermore, active compound from *S. mahagoni* exhibit comparable amino acid residue interactions to those of the control ligand (Table 6).

Table 6. Binding affinity and amino acid residue between compounds from *S. mahagoni* leave ethanolic extract and AKT-1 or MDM2

Ligand	AKT-1		MDM2	
	Binding Affinity	**Amino acid residue**	**Binding Affinity**	**Amino acid residue**
(+)-ar-Turmerone	-6.5	MET227 ; ALA177; VAL164; MET281; LEU156; LYS179	-7.3	ILE19; TYR100; HIS96; LEU54; GLY58; PHE86; LEU57; ILE61; VAL93; ILE99; PHE91
Quercitrin	-7.8	GLY162; LYS158; GLU234; MET281; VAL164; ALA177	-7.5	LEU54; VAL93; ILE61; ILE99; HIS96
Hyperoside	-8	PHE161; LYS158; VAL164; GLU278; GLY157	-6.9	PHE55; GLY58; LEU54; ILE61; ILE99; VAL93 ; GLN72
Imidazole			-9.6	HIS96; LEU54; ILE99; LEU57; PHE91; PHE86; ILE61; VAL93
AZD5363	-9.1	GLU278; GLU234; MET281; ALA230; ASN279; GLY157; MET227; LEU181; LYS179; VAL164; LEU156; ALA177; GLY162		

Note : Glu is glutamate; Met is methionine; Phe is phenylalanine; Ile is leucine; Ala is alanine; Lys is lysine; His is histidine; Leu is leucine; Asn is Asparagine; Val is valine; Gly is glycine; Gln is glutamine; and Try is tyrosine. Underline is mean comparable amino acid residue interactions to the control ligand.

Further evaluation of the compounds' orientation when interacting with the active sites of AKT-1 and MDM2 (Figures 1 and 2, respectively) is crucial for assessing their potential as inhibitors. The analysis indicated that these compounds from the *S. mahagoni* ethanolic extract bind to the active sites of AKT-1 and MDM2 similarly to the known inhibitors, suggesting that Quercitrin, (+)-ar-Turmerone, and Hyperoside could be promising candidates for inhibiting these proteins (Table 6).

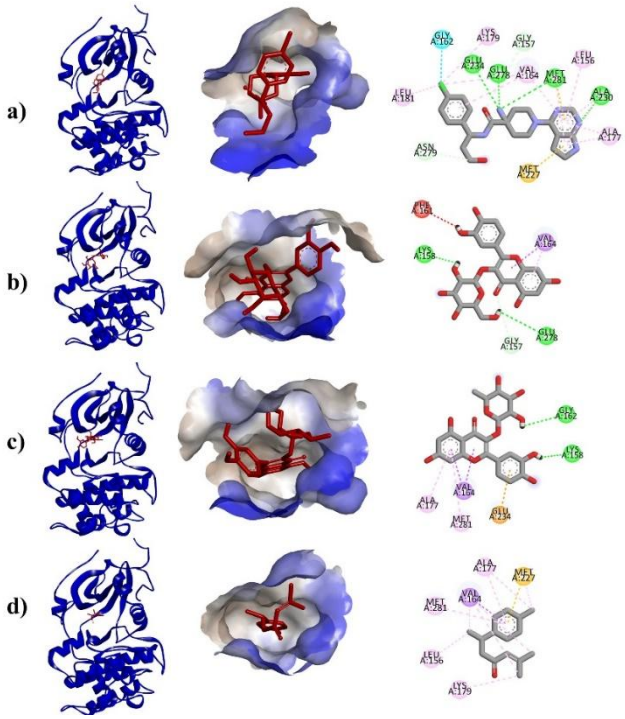


Figure 1. Interaction between AKT-1 and compounds from *S. mahagoni* leaves ethanolic extract. a) docking of AZD5363 as native ligand inhibitor; b) docking of hyperoside; c) docking of quercitrin; d) docking of (+)-ar-Turmerone. On the left, AKT-1 is presented in blue ribbon structure with ligands presented as red. In the middle, hydrophobicity surface map of the active site of AKT-1 with the ligands presented as red cylinders. At the right, cylinder representation 2D interaction between AKT-1 and ligands

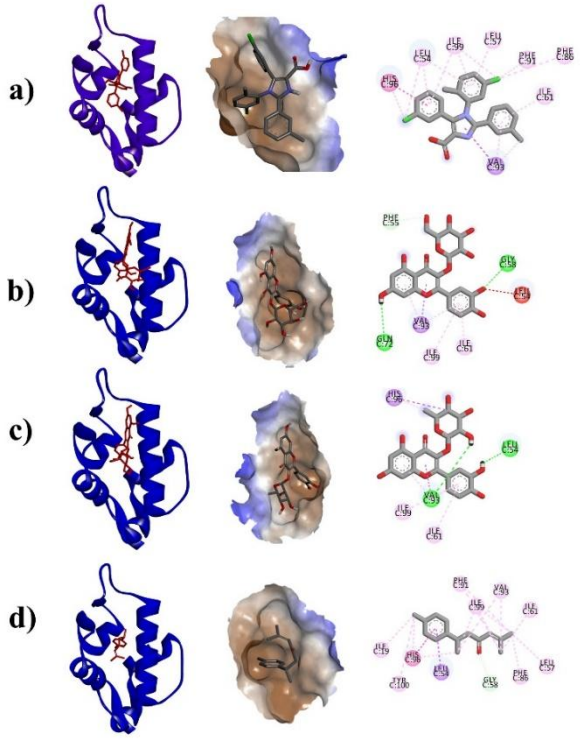


Figure 2. Interaction between MDM2 and compounds from *S. mahagoni* leaves ethanolic extract. a) docking of imidazole as native ligand inhibitor; b) docking of hyperoside; c) docking of quercitrin; d) docking of (+)-ar-Turmerone. On the left, MDM2 is presented in blue ribbon structure with ligands presented as red. In the middle, the hydrophobicity surface map of the active site of MDM2 with the ligands presented as red cylinders. At the right, cylinder representation 2D interaction between MDM2 and ligands

4. DISCUSSION

This study evaluated and predicted the active compound that is contained in *S. Mahagoni* ethanolic extract and their potency for inhibiting cancer cell growth. Phytochemicals are natural bioactive compounds found in plants, such as medicinal plants, which have work act as a defense system against diseases. They exhibit extensive potential as antioxidants, anticancer, anti-inflammatory, and immunomodulator in the body system. In this study, present that *S. Mahagoni* ethanolic extract has potency as antioxidant that have been proven their capability to inhibiting DPPH free radical. This study was supported by another research that another part of *S. Mahagoni* plant, such as seed and bark, also has been reported have potency to inhibiting DPPH free radical [17,20].

Furthermore, In the study revealed a presence of flavonoids and phenolic in the *S. Mahagoni* ethanolic extract, as TFC 458.91 ± 21.60 (mgQE/g w.b) and TPC 299.90 ± 14.28 (mgGAE/g w.b), respectively. The result of this study about TFC and TPC of *S. Mahagoni* leave ethanolic extract was supported by another study about *S. Mahagoni*. 24 compounds were characterized by the GC-MS, and were grouped into phenolics, fatty acids and hydrocarbons, and terpenoids [18] was found in *Mahagoni* leave methanolic extract. Another part of *S. Mahagoni* plant, such as seed and bark, also has been reported contain the phytochemicals [21–24].

Flavonoids and phenolics are classes of compounds commonly referred to as plant secondary metabolites. They are characterized by an aromatic ring with at least one hydroxyl group. Over 8,000 naturally occurring phenolic compounds derived from plants have been documented. Besides that, screening single compounds needs to be conducted to know the specific compound that is contained in this extract. Additionally, further screening showed that this extract contains several plant secondary metabolites, including Quercitrin, (+)-ar-Turmerone, and Hyperoside. These compounds have been reported to have several pharmaceutical function, such as antiinlamatory for quercitrin [25], anticancer for (+)-ar-Turmerone [26], and anti-cancer, brain-protective, neuroprotective, cardioprotective and renal-protective activities for hyperoside [27].

Chemicals intended for use as oral drugs must possess specific characteristics that align with the criteria for drug-likeness. There are two primary types of analyses used to evaluate drug-likeness: Lipinski's Rule and ADME prediction. Lipinski's Rule identifies key physicochemical properties common to drug-like compounds, while ADME prediction evaluates Absorption, Distribution, Metabolism, and Excretion, as well as Toxicity (ADME). This analysis provides insights into oral bioavailability, cellular permeability, metabolism, elimination, and toxicity, which are crucial for understanding a drug molecule's pharmacokinetics and pharmacodynamics. Bioactive compounds isolated from plants can be considered lead compounds if they exhibit a favorable ADME profile. Even if these compounds do not always meet standardized ADME criteria, modifications or substitutions with similar compounds or herbs may still achieve the desired therapeutic effects in herbal medicine.

In the absorption parameter, two critical factors are water solubility and intestinal absorption. Water solubility is essential for drug bioavailability. The ESOL model is commonly used to categorize solubility based on a logarithmic scale (Insoluble <-10, Poorly soluble <-6, Moderately soluble <-4, Soluble <-2, Very soluble <0) [15]. In this study, the water solubility of the three compounds falls within the Moderately soluble to Soluble range. (+)-ar-Turmerone shows a high intestinal absorption value of 94.489%, indicating excellent absorption, whereas Hyperoside and Quercitrin have lower intestinal absorption values of 47.999% and 52.709%, respectively. Optimal intestinal absorption is typically above 80% [28].

The prediction of a drug's or lead compound's distribution within the body is assessed by its volume of distribution, which typically ranges from 0.5 to 3 L/kg.

Quercitrin, (+)-ar-Turmerone, and Hyperoside exhibit relatively favorable drug delivery characteristics in the bloodstream. The metabolism of a drug involves evaluating whether it can inhibit CYP (Cytochrome P450) enzymes, which are crucial for the digestive system and Phase 1 metabolic processes. Quercitrin, (+)-ar-Turmerone, and Hyperoside do not inhibit these CYP enzymes. The final pharmacokinetic parameter to consider is the excretory system. Faster excretion rates lead to higher total clearance values, which generally benefit the body.

Drug-likeness analysis often follows Lipinski's Rule, which provides criteria for evaluating chemical and physical properties necessary for a compound to be considered a viable drug. Lipinski's Rule includes: Log P less than 5, fewer than 10 hydrogen bond acceptors, fewer than 10 hydrogen bond donors, and a molecular mass under 500 Dalton [29]. Compounds from *S. mahagoni* ethanolic extract typically have up to two violations of this rule. According to Lipinski's Rule, a compound is suitable for oral administration if it has no more than one violation. Compounds with two or more violations usually exhibit poor solubility and permeability [30].

This study also aimed to predict the potential of compounds from *S. mahagoni* leaves ethanolic extract in inhibiting cancer cell growth through molecular docking. The binding affinity of Quercitrin, (+)-ar-Turmerone, and Hyperoside with MDM2 and AKT-1 was evaluated. Both MDM2 and AKT-1 are targets in cancer therapy. MDM2 is involved in the degradation of p53, a pro-apoptotic protein , while AKT-1 is crucial for various cellular processes including growth, proliferation, survival, and angiogenesis [31,32]. The findings suggest that Quercitrin, (+)-ar-Turmerone, and Hyperoside may have potential as inhibitors of MDM2 or AKT-1. However, further study, especially in vitro study, should be conducted to validate the activity of the studied compounds.

CONCLUSION

The current study indicates that the ethanolic extract of *S. mahagoni* leaves possesses antioxidant properties, attributable to its content of various phenolic and flavonoid compounds, including Quercitrin, (+)-ar-Turmerone, and Hyperoside. These compounds not only meet Lipinski's criteria for drug-likeness but also show potential as inhibitors of MDM2 and AKT-1. Moreover, Quercitrin, (+)-ar-Turmerone, and Hyperoside might inhibit MDM2 and CXCR4, potentially inducing apoptosis in cancer cells. Further in vitro research is needed to validate these compounds' efficacy and confirm their potential therapeutic applications.

Conflict of Interest

Authors declare that there is no have potential conflict of interest

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سويتينيا ماهاغوني يترك المستخلص الإيثانولي: نشاط مضاد للأكسدة في المختبر، وتحديد المركب النشط والتنبؤ بالسيليكو كمثبط بروتين AKT−1 وMDM2

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ملخص

يدعم الارتباط الوثيق بين الممارسات التقليدية والخصائص الدوائية لهذه النباتات استمرار استخدامها في علاج مختلف الحالات الصحية .قيّمت هذه الدراسة وتوقعت المركب النشط في المستخلص الإيثانولي لأوراق سويتينيا ماهاغوني وفعاليته في تثبيط نمو الخلايا السرطانية .شمل التحليل قياس تثبيط الجذور الحرة DPPH، ومحتوى الفينول والفلافونويد الكلي، وتقييم تشابه الأدوية، ودراسات الالتحام الجزيئي .تشير النتائج إلى أن المستخلص الإيثانولي لأوراق S. mahagoni يُظهر خصائص مضادة للأكسدة نظرًا لاحتوائه على مركبات فينولية وفلافونويدية مثل الكيرسيترين، و(+)-ar-Turmerone، وهايبروسيد، والتي تُلبي أيضًا معايير ليبينسكي .بالإضافة إلى ذلك، قد تعمل هذه المركبات كمثبطات لـ MDM2أو AKT-1، مما قد يُعيق MDM2وAKT-1 ويُحفز موت الخلايا المبرمج في الخلايا السرطانية .ينبغي إجراء المزيد من الأبحاث في المختبر للتحقق من فعالية المركبات المدروسة.

الكلمات الدالة: مضاد للسرطان، Insilico، دواء نباتي، مستقلب ثانوي، Swietenia mahagoni.

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Cigarette Smoking Influences Montelukast Pharmacokinetics in Jordanian Population

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ABSTRACT

Background: Montelukast is one of the main therapeutic agents used for asthma management. Its therapeutic effectiveness is greatly influenced by the expression of metabolic enzymes and/or transporters involved in its disposition.

Objectives: To assess the effect of smoking on montelukast pharmacokinetics in four bioequivalence studies against the reference drug Singulair®.

Methodology: Data were extracted from bioequivalence studies to compare 10 mg generic Montelukast to Singulair® the originator. Primary pharmacokinetic parameters, maximum plasma concentration (C_{max}) and area under the curve ($AUC_{0\text{-}inf}$ and $AUC_{0\text{-}t}$) were calculated using Kinetica®. Analysis of Variance was performed to compare montelukast pharmacokinetics between smokers and non-smokers. Statistical significance was set at $P \leq 0.05$.

Results: Mean± SD montelukast C_{max} (ng/mL) was 397.1 ± 125.7 in non-smokers compared to 352.8± 133.9 in smokers. Significant alterations in montelukast C_{max} (P= 0.0206), AUC $_{0\text{-}t}$ (ng. h/L) 2335 ± 111, P= 0.0016, and AUC $_{0\text{-}inf}$ (ng. h/L) 2509 ± 1163, P= 0.0015 were observed in the study participants who are smokers.

Conclusion: Despite the minimal fold-decrease in montelukast pharmacokinetic parameters in smokers compared to non-smokers, this might have a profound clinical impact on the therapeutic effectiveness of montelukast in patients.

Keywords: Montelukast Pharmacokinetics, smoking, bioequivalence, Singulair®, Montelukast bioequivalence, enzyme induction.

INTRODUCTION

Montelukast is a cysteinyl leukotriene subtype 1 receptor antagonist that has demonstrated high efficacy for allergic rhinitis and asthma treatment [1]. It was first licensed in 1998 under the brand name Singulair® and is available in three different forms; 4 mg oral granules, 4 and 5 mg chewable tablets and 10 mg film-coated tablets [2, 3]. In healthy adults, montelukast reaches the maximum plasma concentration (C_{max}) in 3–4 h following administration of 10 mg. *In vitro* analysis of montelukast metabolism showed major involvement of CYP3A4, CYP2C8, CYP2C9, and UGT1A3 enzymes [4]. Average elimination half-life ($t_{1/2}$) is 2.7 to 5.5 hours with no

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gender, age and body mass index (BMI) significant differences in Montelukast pharmacokinetics [5, 6].

Different studies comparing montelukast generics to Singulair® demonstrated bioequivalence under both fasted and fed states [7, 8]. However, adverse events have been reported when montelukast was administered under fasting conditions [7].

According to *Angelica Tiotiu et al*., 20% of patients with asthma are cigarette smokers [9]. The same study reported poor asthma control and higher exacerbation of symptoms in patients who were identified as current smokers [9]. Additionally, a poor response to corticosteroid treatment has been observed in this group of patients [10]. Unfortunately, the effect of smoking on treatment plans and outcomes is underestimated and has not received proper attention [11, 12]. For instance, smokers are excluded from pivotal clinical trials, which eventually leads to misinterpretation of the outcome in the general population, bearing in mind the high prevalence of smoking habits.

The objective of this study was to evaluate the effect of smoking habits on montelukast pharmacokinetics in four different bioequivalence studies.

METHODOLOGY

Materials:

Ethical approvals: The Institutional Review Board (IRBs) granted approval for this study on 07/06/2008, and the study was carried out under the protocol study number 32-16122-09-4223. The study precisely followed the ethical guidelines of the Declaration of ICH-GCP, Helsinki, and Jordan Food and Drug Administration, confirming that the participants were fully aware and consented before participation. The Investigator Good Clinical Practice (GCP) statement manages ethical conduct, stresses regulatory agreement, and protects participants.

Instrumentation and chromatographic conditions

The LC-MS/MS system used was an Agilent 1200 series (Agilent Technologies, India) equipped with a G1311A quaternary pump, which was attached to an API 4000 detector from SCIEX Applied Biosystems/MDS. Chromatograms were obtained using Analyst software (version 1.6). Chromatographic separation was carried out on a Thermo Hypersil GOLD™ Cyano HPLC column (50 × 4.6 mm; 5 µm) at 20 °C. Separation was achieved using an isocratic mobile phase consisting of 0.5 mM ammonium chloride and acetonitrile (20:80% v/v) at a flow rate of 0.5 mL/min. The total sample run time was 0.7 minutes (Figure 1). Detection of Montelukast and Montelukast-D6 (internal standard) was achieved using multiple reaction monitoring (MRM) in the positive ionization mode under optimized conditions, as summarized in Tables 1 and 2.

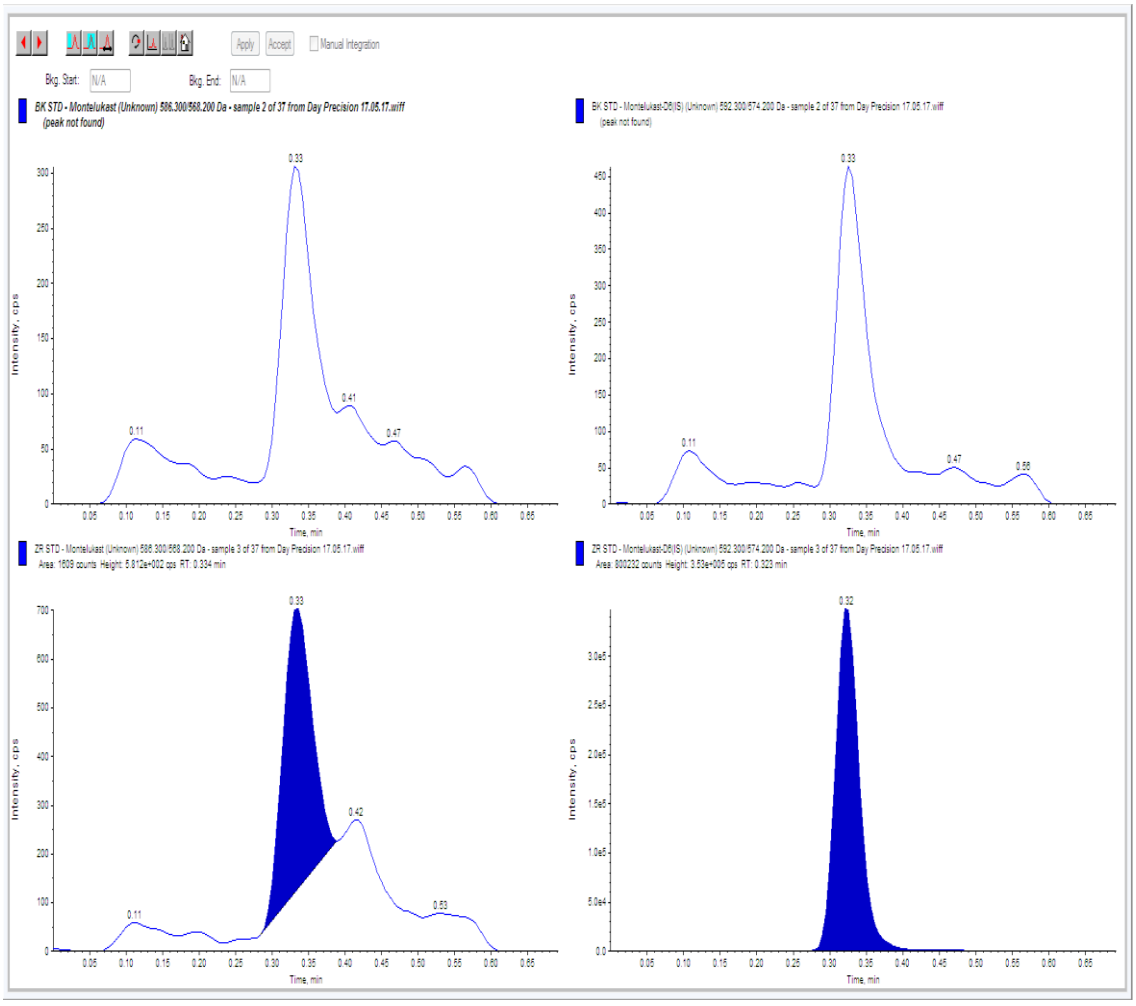
Table 1: Summary Table of Chromatographic Conditions and Mass Spectrometric Conditions

Flow Rate	**0.550 mL/min**
Column Temperature	20 °C
Autosampler Temperature	10 °C
Injection Volume	5 µl
Total Run Time	0.7 min
Column	Thermo Hypersil GOLD CN, (50×2.1) mm , 5 µm

Table 2: Compound's detection and retention times:

Compound Name	**Detection**			**Retention Time**	
Montelukast	Parent 586.30 and daughter 568.20			0.33 min	
Montelukast_D$_6$	Parent 592.30 and daughter 574.20			0.33 min	
MRM Parameters					
Compound Name		**DP**	**EP**	**CE**	**CXP**
Montelukast		81.0	10.0	19.0	22.0
Montelukast_D$_6$		81.0	10.0	19.0	22.0
Positive Mode					
CUR	**CAD**	**GS1**	**GS2**	**Temp.**	**IS Voltage**
25	8	35	50	550	5500

A



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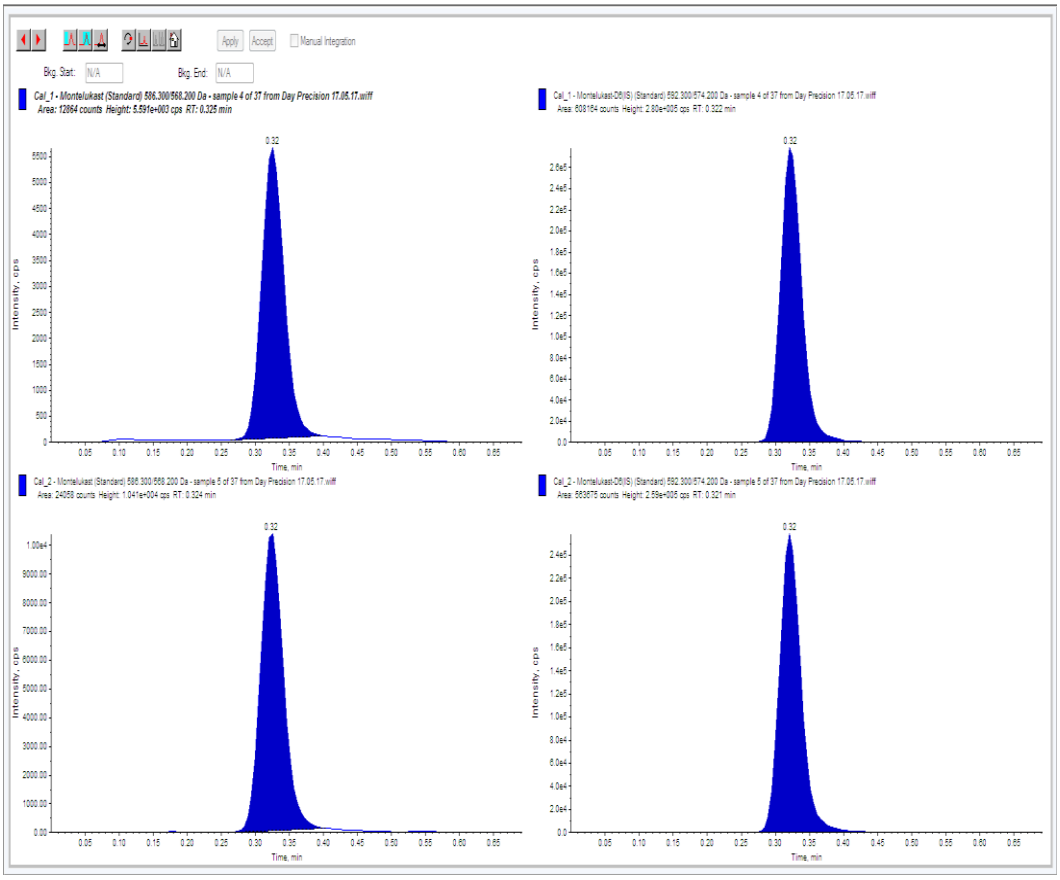

Figure 1: Representative MRM chromatograms of (A) Montelukast chromatograms for blank plasma free of Montelukast or IS and zero standard (Blank plasma with IS), (B) Montelukast HPLC chromatograms, LLOQ Blank plasma spiked with Montelukast (1st Calibrator 10.0 ng/ml,) and 2nd Calibrator 20.0 ng/mL.

Montelukast working solutions: 10 mg of montelukast was dissolved in 7 mL of methanol (MeOH) in 10 mL of V.F, vortexed until dissolved, and completed to volume with MeOH, to obtain a concentration of (1.000 mg Montelukast/mL) stock solution. Five hundred microliters of montelukast stock solution (1.000 mg/mL) were diluted in 10 mL of (1:1 methanol: water). The final concentration was (50.000 µg Montelukast/mL). Montelukast_D_6 was dissolved in an equal volume of MeOH to obtain a concentration (1000 mg Montelukast_D_6 /mL). Montelukast_D_6 stock solution (25 µL, 1.000 mg/mL) was diluted to 10.0 ml of (1:1 methanol: water). The concentration obtained was (2.500 µg of Montelukast_D_6 /mL).

Method validation: The LC-MS/MS method was developed and validated according to the International Committee for Harmonization (ICH) guidelines. Interday accuracy, precision, linearity, recovery, stability, and robustness were assessed. The method linearity was investigated in the range of 10.00 -600.00 ng/mL. Method validation was previously published by *Said et al* [13].

Conduct of the bioequivalence study: Four separate

open, randomized, single-dose, two-way crossover bioequivalence studies were performed for four different test formulations to compare their bioavailability to that of the reference drug Singulair® 10 mg tablet. All studies were performed according to the GCP guidelines and were approved by the Jordan Food and Drug Administration. The main inclusion criteria were being healthy, male and aging 18-45 years. IRB approval and consent forms were obtained prior to study initiation and dosing. Participants were randomized to be offered either the reference drug, Singulair® 10 mg tablet or the test drug of the same strength. All participants received references and tests in either period 1 or period II. Participants fasted for 10 h prior to montelukast administration. The tablet contained 240 mL of ambient water. Blood samples were collected prior to dose administration and up to 24 h post-dose into K2EDTA tubes. The time points of samples collection were pre-dose, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.33, 2.66, 3, 3.33, 3.50, 4, 5, 6, 8, 10, 12 and 24 hours. Blood samples were stored at -80 °C until the time of bioanalysis. Plasma samples were obtained *via* centrifugation for 10 minutes at 4000 RPM. The montelukast concentration was measured using the validated LC-MS/MS method. A total number of blood samples were collected and analyzed were 6912 samples and no withdrawal or dropouts were reported.

Pharmacokinetic and statistical analyses: The primary pharmacokinetic parameters, $AUC_{0\text{-}inf}$, $AUC_{0\text{-}t}$, and C_{max} were calculated using non-compartmental analysis (Kinetica® 2000 version 4.1, Innaphase Corporation, France). A 90% confidence interval for the intra-individual ratios (test/reference) of the primary pharmacokinetic parameters was calculated, and an acceptance criterion for bioequivalence was set at (80%-125%). The significance level was set at $P < 0.05$. Unpaired Student's *t*-test was performed using GraphPad Prism version 6.01, released in 2012 (GraphPad Software, San Diego, USA), to compare the two formulations and investigate the period, subject, formulation, and sequence effects. Other covariates, such as demographic data (age, sex, height, weight, and smoking status), were also incorporated into the model. Univariate and multivariate regression analyses were used to assess the association between demographic data, lifestyle habits, and montelukast primary pharmacokinetic parameters. Data were summed from the four studies after ensuring that the same inclusion/exclusion criteria were maintained, IRB and consent forms were included, and sampling times were consistent.

RESULTS

Validation of the LC-MS/MS method

The analytical method was fully validated, including all critical parameters, such as accuracy, precision, specificity, linearity, stability, matrix effect, and robustness. Each parameter was fully evaluated to ensure the reliability and suitability of the method for the anticipated application [13]. The method was linear in the range 10.00 -600.00 ng/mL. The method proved to be precise and accurate; interday - intraday precision and accuracy were with CV% less than 8%, which is acceptable according to the ICH guidelines. The stability of the method was found to be consistent and reliable under various conditions, such as long term, short term, and room temperature. Short-term stability was assessed at different concentrations by comparing the analyzed quality control samples with their supposed concentrations, and the results showed that the samples remained stable for 18 h. Long-term stability was measured using QC samples kept at -70 ◦C for 31 days at different concentrations (LQC and HQC levels), and no significant concentration difference was observed, implying the stability of Montelukast and Montelukas_D6 at both -20 ± 5 ◦C and -70 ± 10 ◦C for a time interval of 31 days. No matrix effect was observed, and an acceptably high recovery was achieved [13].

Montelukast pharmacokinetics

A total of 192 subjects were analyzed. The study participants were classified according to smoking status, and 56.25% were smokers. Mean± SD age and BMI were 29.86 ± 5.77 years and 26.02 ± 2.44 kg/m^2, respectively in non-smokers group. On the other hand, smokers had mean ± SD age and BMI of 29.26 ± 5.10 years and 25.34 ± 2.56

kg/m^2, respectively. Age and BMI were not predictors of montelukast pharmacokinetics according to smoking status (P= 0.4659 and P= 0.0643, respectively).

The plasma concentration vs. time profiles of both the test formulations and Singulair® were comparable (Figure 2). Mean± SD C_{max} and t_{max} of Montelukast for the test formula were 377.44± 124.06 ng/mL and 3.4± 1.36 hr while for the reference 369.29± 134.45 ng/mL and 3.21± 1.18 hr. The AUC_{0-t} was 2551.84± 1101.07 ng.hr/mL for the reference compared to 2580.19± 979.92 ng.hr/mL in the test group whereas AUC_{0-inf} was 2742.78± 1142.62 ng.hr/mL and 2767.48± 1073.95 ng.hr/mL in the reference and test groups, respectively. The study participants who were smokers had a significantly lower C_{max} than non-smokers by 11.16% (P= 0.0206) (Table 3). No significant difference in t_{max} was observed between smokers and non-smokers (P= 0.4065). In contrast, AUC $_{0-t}$ and AUC $_{0-inf}$ were significantly different between the two groups (P= 0.0016 and P= 0.0015, respectively). The fold-change in both AUC $_{0-t}$ and AUC $_{0-inf}$ was approximately 0.82-folds in smokers compared to that in non-smokers. Similarly, C_{max} in smokers group was 0.89 of that in non-smokers group (Table 3).

Table 3: Demographic and pharmacokinetic data of Montelukast presented as mean± SD

Variable	Non-smoker	Smoker	Difference (Smoker – Non)	95% CI	P-Value
N	84	108			
Age (Years)	29.86 ± 5.77	29.26 ± 5.10	-0.60 ± 0.82	-2.21 to 1.02	0.4659
BMI (kg/m^2)	26.02 ± 2.44	25.34 ± 2.56	-0.68 ± 0.36	-1.4 to 0.04	0.0643
C_{max} (ng/mL)	397.1± 125.7	352.8 ± 133.9*	-44.3 ± 18.9	-81.7 to -6.88	0.0206*
T_{max} (hours)	3.25 ± 1.42	3.41 ± 1.22	0.16 ± 0.19	-0.22 to 0.54	0.4065
AUC $_{0-t}$ (ng.hr/L)	2821 ± 953	2335 ± 111**	-486 ± 152	-785 to -186	0.0016**
AUC $_{0-inf}$ (ng.hr/L)	3020 ± 989	2509 ± 1163**	-511 ± 157	-823 to -198	0.0015**

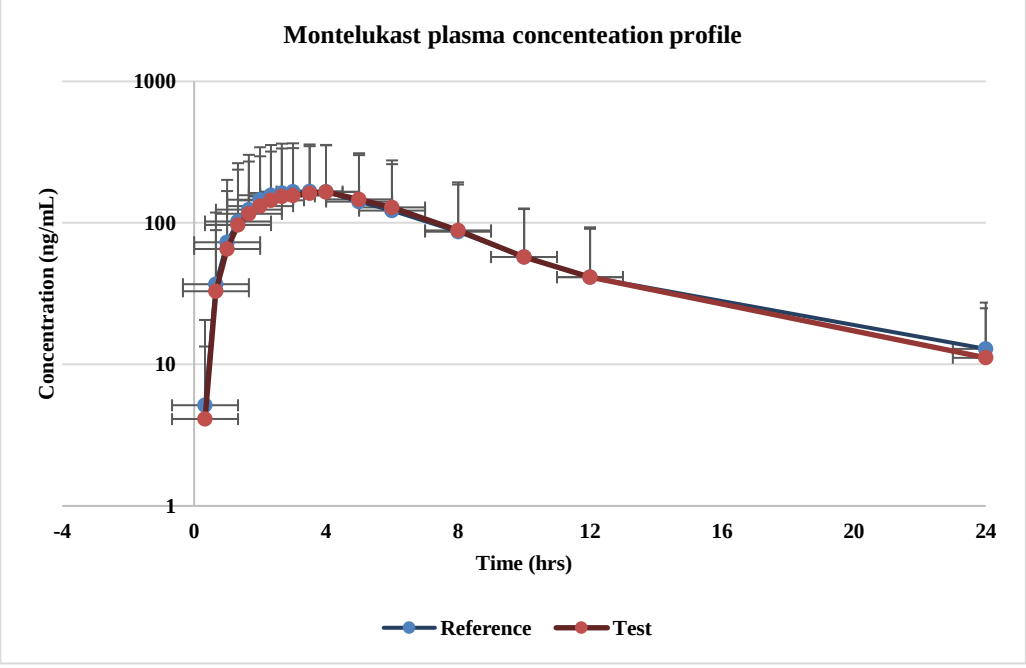

Figure 2 depicts the pharmacokinetic plasma concentration vs. time profile of both the test and reference (Singulair®) formulations of montelukast from four different bioequivalence studies (n=192).

DISCUSSION

Montelukast is one of the main therapeutic agents used for asthma treatment. Several formulations that are bioequivalent to the originator Singulair® are available in the market. However, therapeutic effectiveness can be influenced by several demographic factors, such as smoking status. Consequently, this study aimed to evaluate the effect of smoking status on montelukast pharmacokinetics in four different bioequivalence studies that compared generic formulations of Montelukast to Singulair®.

In the current study, smokers had a significantly reduced montelukast C_{max} compared to non-smokers (P= 0.0206). Similar findings were observed for amitriptyline, clozapine, and mirtazapine pharmacokinetics in smokers [14]. This could be explained by the demonstrated effects of cigarette smoking on the metabolism of different therapeutic agents. These effects are due to the induction of metabolic enzymes, whether phase I or Phase II enzymes [15]. For instance, clozapine and olanzapine pharmacokinetics significantly being influenced by cigarette smoking [16, 17]. Similar findings were reported for theophylline clearance, which increased in smokers (0.063 ±0.019 L/h/kg) compared with 0.040 ± 0.008 L/h/kg [18].

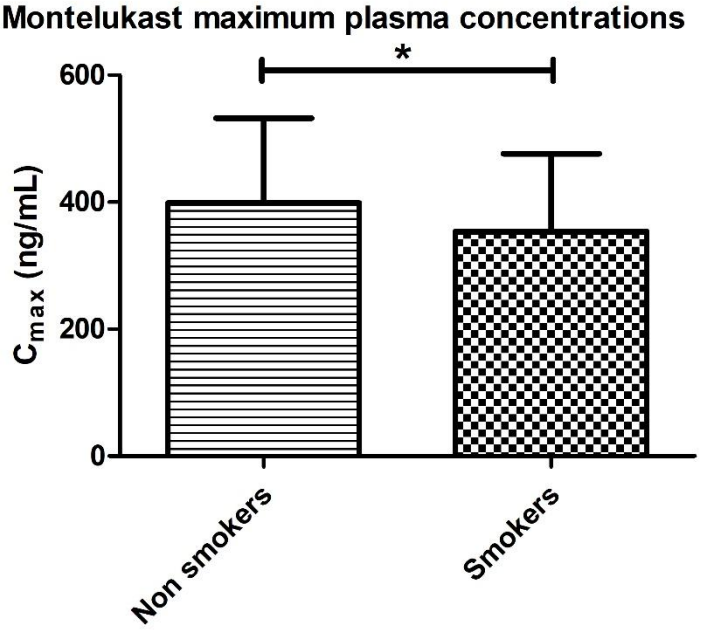

Figure 3 shows the differences in Montelukast C_{max} between smokers and nonsmokers. * Statistical significance.

Previous studies have reported the ability of cigarette smoking to induce the expression of CYP450 enzymes and drug transporters such as OATP1B1, OATP2B1, OAT2, NTCP, OCT1, and BSEP *via* aryl hydrocarbon receptor activation [19]. Montelukast hepatobiliary elimination is mediated through OATP1B1 transport, in which genetic polymorphisms in *SLCO2B1* gene coding for OATP2B1, such as rs12422149, are significantly associated with reduced plasma concentrations of montelukast [20, 21]. This could potentially influence the therapeutic efficacy of montelukast, especially in patients with asthma [20]. Thus, patients who are smokers and use montelukast to manage their asthma might need dosage adjustment and/or monitoring to achieve therapeutic effectiveness of their treatment. Nicotine, the

main ingredient in cigarettes, was found to induce the UGT1A3 enzyme which is one of the main enzymes involved in montelukast metabolism [14]. CYP2C9 polymorphisms may play a major role in altering montelukast pharmacokinetics [22]. Further pharmacogenetic studies are required to investigate the association between cigarette smoking, genetic polymorphisms, and montelukast pharmacokinetics.

Management of asthma primarily depends on the therapeutic concentrations achieved following administration as well as adherence to medication. Considering that one-fifth of asthmatic patients are smokers and montelukast is a major therapeutic agent in their regimen, patients should be monitored for asthma management if they are smokers. A rigorous judgment on the need to adjust the montelukast dose in smokers cannot be made without performing a clinical trial on patients with asthma who are smokers. Previous studies were performed on the montelukast and fluticasone combination compared to placebo. The results showed improved asthma management in asthmatic smokers. However, the smoking effect was not investigated in this trial as a covariate; thus, no concrete conclusions can be drawn [23].

A limitation of this study is that the data were taken collectively from four different bioequivalence studies despite having the same inclusion/exclusion criteria and being from the same ethnic background. Additionally, the pharmacokinetics of these four formulations might be different owing to differences in formulation. However, this limitation was found in both the reference and test groups, which could limit bias.

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Conflict of interest

The authors have no conflict of interest to declare

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Authors' contribution

Dr. Rana Said was the primary investigator who designed and conceptualized the study. Rana Abutaima interpreted the data and drafted the manuscript. Dr. Lidia K. Al-Halaseh and Dr. Khaldun have reviewed the manuscript. Dr. Basel Arafat and Prof. Tawfiq Arafat supervised the bioequivalent studies.

Data availability

All data generated in this study is available within the manuscript or supporting material.

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تأثير تدخين السجائر على الحرائك الدوائية لمونتلوكاست في السكان الأردنيين

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ملخص

المقدمة: يُعد مونتلوكاست أحد الخيارات العلاجية الرئيسية المستخدمة في علاج الربو. تتأثر فعاليته العلاجية بشكل كبير بتعبير الإنزيمات الأيضية و/أو النواقل المشاركة في توزيعه.

الأهداف: تقييم الاختلافات في الحرائك الدوائية لمونتلوكاست في أربع دراسات تكافؤ حيوي مقارنةً بالدواء المرجعي سينجولير®.

المنهجية: تم جمع البيانات بشكل رجعي من دراسات التكافؤ الحيوي لمقارنة تحضيرات مونتلوكاست الجنيسة بجرعة 10 مجم مع دواء سينجولير® الأصلي. تم حساب المعلومات الدوائية الأساسية؛ تركيز البلازما الأقصى، المساحة تحت المنحنى AUC_{0-inf} و AUC_{0-t} باستخدام برنامج Kinetica®. تم إجراء تحليل التباين (ANOVA) لمقارنة الحرائك الدوائية لمونتلوكاست بين المدخنين وغير المدخنين. تم اعتبار قيمة $P \leq 0.05$ دلالة إحصائية.

النتائج: كان متوسط ± الانحراف المعياري للعمر ومؤشر كتلة الجسم 5.10 ± 29.26 سنة و25.34 ± 2.56 كجم/م^2 في مجموعة المدخنين. كان تركيز مونتلوكاست الأقصى 125.7 ± 397.1 (ng/mL) في غير المدخنين مقارنةً بـ 352.8 ± 133.9عند المدخنين. تم ملاحظة تغيير كبير في تركيز مونتلوكاست الأقصى(P= 0.0206) ، و AUC_{0-t} (ng.hr/L) (P= 0.0016), 2335 ± 111و AUC_{0-inf} (ng.hr/L) (P= 0.0015), 2509 ± 1163 في المشاركين.

الاستنتاج: بالرغم من الانخفاض الطفيف في معلمات الحرائك الدوائية لمونتلوكاست لدى المدخنين مقارنةً بغير المدخنين، إلا أن ذلك قد يكون له تأثير سريري كبير على الفعالية العلاجية لمونتلوكاست لدى المرضى الذين يستخدمونه.

الكلمات الدالة: الحرائك الدوائية لمونتلوكاست، التدخين، التكافؤ الحيوي، سينجولير®، التكافؤ الحيوي لمونتلوكاست، تحفيز الإنزيمات.

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The Phenolic Content of Syrian *styrax officinalis* L. Fruits and its Antioxidant and Anti-Cholesterol Activities

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ABSTRACT

The objective of the current study was to determine the chemical constituents, total phenols, antioxidant activity, and hypocholesterol effect of the aqueous and ethanolic extracts of *Styrax officinalis* fruits.The chemical composition of the extracts was assessed by (GC) and (GC-MS). The extract contained various compounds, with styracitol (90.8-97.4%) being the major compound in the aqueous and ethanolic extracts, respectively. The total phenolic content was quantitatively determined using the Folin-Ciocalteu reagent, with gallic acid as the standard. Antioxidant activity was assessed by measuring the scavenging of 2,2-Diphenyl-1-Picryl-Hydrazyl (DPPH). The total phenolic content of the aqueous and ethanolic extracts was found to be 19.2 ± 0.75 mg GAE/g and 9.16 ± 0.23 mg GAE/g, respectively, in terms of gallic acid equivalent (GAE). The antioxidant activity has an IC50 of 1.076-2.49 mg/mL, respectively. The anti-cholesterol activity was determined using the CHOD-PAP method. The aqueous extract was more effective than the ethanolic extract at lowering cholesterol. The IC_{50} for the ethanolic extract was 6.4 mg/ml, while the IC_{50} for the aqueous extract was 0.54 mg/ml.

Keywords: *Styrax officinalis* ; Phenols ; Antioxidant ; Anti-cholesterol ; CHOD/PAP.

1. INTRODUCTION

Medicinal plants have been used in healthcare for many years. They play important roles in disease prevention and promotion of health. Medicinal plants contain natural compounds that are important sources of molecules with medicinal properties [(1)].

Styrax.officinalis L. is an important medicinal plant that grows in desert, temperate climate, subtropical, and Mediterranean Basin regions. It has been used for medicinal, cosmetic, and agricultural purposes [(2,3)]

Earlier studies on the pericarps of Styrax species have reported the isolation of a triterpene sapogenin and a triterpene saponin [(4)], Which has cholesterol-lowering efficacy [(5,6)]

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Phenolic compounds contain hydroxyl groups that directly contribute to antioxidant action, and also some of these compounds stimulate the synthesis of endogenous antioxidant molecules within the cell. After reviewing the literature, it was found that, phenolic compounds have been found to demonstrate free radical inhibition, metal inactivation, peroxide decomposition, or oxygen scavenging in biological systems, thereby aiding in the prevention of oxidative diseases [(7)] .Therefore, it is essential to investigate the phenolic content and antioxidant activity of different plants.

Hypercholesterolemia, known as high cholesterol levels, is responsible for one-third of cases of ischemic (coronary) heart disease worldwide.

Hypercholesterolemia causes 2.6 million deaths (4.5% of the total) and results in 29.7 million disability-adjusted life years (DALYs), accounting for 2.0% of the total DALYs. The worldwide prevalence of high total

cholesterol among adults in 2008 was 39%, with 37% in men and 40% in women [8].

The use of medicinal plants in combating non-communicable diseases has been extensively documented through field studies worldwide. However, a systematic compilation of these surveys for specific disease conditions, such as hypercholesterolemia, has not been carried out [9].

According to previous literature, there are four mechanisms through which bioactive compounds from herbal medicines reduce lipid levels. These mechanisms include inhibiting the absorption of cholesterol in enterocytes, reducing cholesterol synthesis, enhancing reverse cholesterol transport, and stimulating cholesterol excretion in the liver [9], so far, there is no study to determine the effectiveness of *S. officinalis* in lowering cholesterol.

2. MATERIALS AND METHODS

2.1. Chemicals

Gallic acid standard was purchased from AvonChem (United Kingdom); Folin–Ciocalteu reagent and ethanol (95%) were obtained from Sigma; 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Tokyo Chemical Industry (Japan); methanol from SHAM LAB; sodium carbonate from Scharlau; and the CHOD-PAP kit from Coral Clinical Systems (India).

2.2. plant samples collection

Fruits of *s. officinalis* were harvested in September 2023 during maturation (seeds: dark brown

Color) from the coastal region (Tartous) of Syria.

The plant was identified by prof. G.TAYOUB (Tishreen university), The voucher specimens were deposited at the herbarium, Damascus University.

Figure 1. ***Styrax officinalis***

2.3. Sample preparation

The seeds from the fresh fruits samples were manually separated from pericarp, then the pericarp where dried on filter paper by oven at 50°C until constant weight.

2.4. Extraction

Pericarps of fruits (50 g) were grounded using a milling machine and divided into two equal samples.

The first was prepared as ethanolic extract using 200 ml of 70% ethanol; the second was dissolved in 200 ml of distilled water. An ultrasound path set 25°C, 2 h was used to dissolve the powder completely.

Followed maceration at room temperature for 48 h, the extract was filtered and dried. Then were stored at 2-8 °C until use.

2.5. Gas Chromatography

Extracts of fruits were analyzed using an Agilent GC system. The capillary column used was BPS (30 m x 0.25 mm) with helium as the carrier gas at 1 ml/min.

The initial temperature of the column was 50°C (held for 5 min), and then heated to 300°C at a rate of 0°C held for 5.5 min. The injector temperature was 300°C with a source temperature of 280°C.

2.6. Gas Chromatography- mass spectroscopy (GC-MS)

Constituents of the extracts were identified using GC-MS. The GC-MS analysis was carried out using an Agilent GC-MS model GC-6890, with an inert mass selective detector 5973.

The capillary column was BP5 (30x0.2 mm, film thickness 0.25 µm). The operating conditions were as follows: carrier gas, helium with a flow rate of 1 mL/min, injected volume was 1 µL of the extract.

The GC-MS system was operated under the following conditions: injection temperature 300°C.

The initial temperature of the column was 50°C (held for 2 minutes), then heated to 170°C at a rate of 2°C/min (held for 7 minutes), and finally heated to 300°C at a rate of 10°C/min (held for 5.5 minutes).

Identification of components in the extract was based on retention time (RT).

Individual components were identified by comparing mass spectra and their corresponding GC retention data. Identification was made by comparing the obtained mass spectra with those in data system libraries installed in the GC-MS system. The quantitative analysis of percentages was performed according to reference materials and standards obtained from Aldrich. Calculations were made using the gas chromatography ChemStation software.

2.7. Determination of phenolic content

The total phenolic content of the extracts was determined colorimetrically by the Folin-Ciocalteu method [10-12]. The F–C reagent was to a 1:10 ration distilled water just before the experiment.

Proceed by taking a 1 mL aliquot of extract and mixing it mixed with 1 mL of Folin-Ciocalteau phenol reagent. After the reaction, 1N Na_2CO_3 was added. The absorbance was measured at a wavelength (λ) of 740 nm using a UV-Vis spectrophotometer.

The standard gallic acid curve was determined by creating five standard solutions of gallic acid with concentrations ranging from 0.05 to 0.6 mg/ml, as shown in **Figure 2A**.

From each concentration of the standard gallic acid solution, 40 µL was taken and mixed with 3.16 ml of distilled water, and 600 µl of 1N Na_2CO_3. The mixture was then shaken and left to stand for 8 minutes and 30 seconds. Afterward, 200 µL of Folin-Ciocalteu reagent was added, and the solution was measured using a UV-Vis spectrophotometer at a wavelength 740 nm.

160 µL of sample, standard, or blank from the assay tube was transferred to a clear 96-well microplate (see **Figure 2B**) and the absorbance of each well was measured at 740 nm.

Figure 2. A- Standard series of gallic acid
B- the Microplate Contains samples and standards

2.8. Antioxidant activity

The sample was diluted into 5 concentrations: (6, 3.75, 2.5, 1.25, 0.5) mg/ml of aqueous extract and (6, 3.25, 2.5, 1.5, 1.25) mg/ml of ethanolic extract. The DPPH radical scavenging activity of *S. officinalis* was assessed using the method described by [13,14] with some modifications. Aliquots of 160 µL of DPPH in methanol were mixed with 40 µL of the extracts. The mixtures were vigorously shaken and then left to stand for 30 minutes at 25 °C under subdued light. The absorbance of each test sample and control sample was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm, as shown in (Figure 3A**)**.

The percentage of DPPH inhibition is calculated using the following equation:

IC (%) = (1 - (As / A0)) * 100

Where A0 represents the absorbance of the negative control, and as represents the absorbance of the sample. After calculating the percentage of inhibition using the above equation, each sample concentration will be determined. This assay is performed in micro centrifuge tubes and assessed using a 96-well plate reader, as shown in (Figure 3B).

Figure 3. A- Microplate contains sample,DPPH
B- UV-Vis-spectrophotometer (made in USA)

2.9. cholesterol-lowering activity

The cholesterol-lowering activity was determined using the CHOD-PAP method [15].

2 mL of a standard cholesterol solution (200 mg/dl) was taken and then added to 2 mL of the sample solution. The mixture was homogenized using ultrasound and then incubated at 37°C for 60-minutes.10 µL of each sample was taken, and then 1000 µL of the CHOD-PAP cholesterol reagent kit was added. The mixture was incubated at 37°C for 10 minutes.200 µL of sample, standard, and blank were

transferred from the assay tube to a clear 96-well microplate (see Figure 4B), and the absorbance of each well was measured at 500 nm using a UV-Vis spectrophotometer.

A separate blank was prepared for each concentration of plant samples. 20 µL of plant samples were mixed with 20 µL of distilled water, according to the experimental conditions. Subsequently, 10 µL was extracted from the mixture, and 1000 µL of reagent was added to eliminate any interference from the plant in the experiment. The inhibition ratio of cholesterol was calculated using the following equation: I% = ((A_STD – A_TEST) / A_STD) x 100

Where A_STD represents the absorbance of standard and A_Test represents the absorbance of Test.

Figure 4. A- Cholesterol SR Kit
B- the Microplate Contains tests and standards.

2.10. Statistical analysis

All tests were performed in three repetitions, and the measurement was repeated three times for each sample. The results were presented as mean ± standard deviation after being calculated using Microsoft Excel 2013. The statistical analysis was conducted using the Costat 6.4 program. The analysis included an analysis of variance (ANOVA) and post-hoc comparisons (LSD) test at a significance level of 0.05.

3. RESULTS AND DISCUSSION

3.1. Phytochemical analysis of the plants extracts

The yielded extracts obtained by ethanolic and aqueous extraction of fruit S.officinalis were about (35%-29%) w/w on dry weight basis, respectively.

The active constituents available in styrax officinalis fruits were identified using GC-MS.

Table 1: Chemical constituents of S. officinalis fruit aqueous extract identified by GC-MS.

Chemical Compound	*Molecular formula*	*Molecular weight*	RT	*Percent %*
Melezitose	***C18H32O16***	***342.30 g/mol***	15.3	0.9%
Paromomycin	***C23H45N5O14***	***615.6 g/mol***	25.52	0.3%
2-Myristynoyl pantetheine	***C25H44N2O5S***	***484.7 g/mol***	25.74	0.1%
DL-Arabinose	$C_5H_{10}O_5$	***150.13 g/mol***	12.95	0.3%
Melibiose	***C12H22O11***	***342.30 g/mol***	12.57	0.2%
Styracitol	***C6H12O5***	***164.16 g/mol***	22.69	90.8%
palmitic acid	***C16H32O2***	***256.42 g/mol***	24.07	0.9%
Vitamin E	***C31H52O3***	***472.7 g/mol***	34.11	0.1%

Table 2: Chemical components of *S. officinalis* fruit ethanolic extract identified by GC-MS.

Chemical Compound	*Molecular formula*	*Molecular weight*	RT	*Percent %*
Melezitose	***C18H32O16***	***342.30 g/mol***	15.5	1.1%
Oleic Acid	***C18H43O2***	***282.5 g/mol***	25.93	0.1%
Paromomycin	***C23H45N5O14***	***615.6 g/mol***	27.35	0.3%
2-Myristynoyl pantetheine	***C25H44N2O5S***	***484.7 g/mol***	26.14	0.1%
DL-Arabinose	$C_5H_{10}O_5$	***150.13 g/mol***	12.95	0.3%
Melibiose	***C12H22O11***	***342.30 g/mol***	15.5	0.2%
Styracitol	***C6H12O5***	***164.16 g/mol***	22.73	97.45%
palmitic acid	***C16H32O2***	***256.42 g/mol***	35.03	0.9%
d-Mannose	***C6H12O6***	***180.16 g/mol***	23.84	0.2%
Vitamin E	***C31H52O3***	***472.7 g/mol***	34.11	0.1%

3.2. Determination of phenolic content

Table 3. Average absorbance values of a series of standard concentration of standard prepared from Gallic acid reference material.

concentration(mg/ml)	0.05	0.1	0.2	0.5	0.6
Average absorbance	0.002	0.016	0.039	0.102	0.124

When graphically representing the relationship between the previous values, the graph of Gallic acid was used to calculate the phenol content (Figure 5).

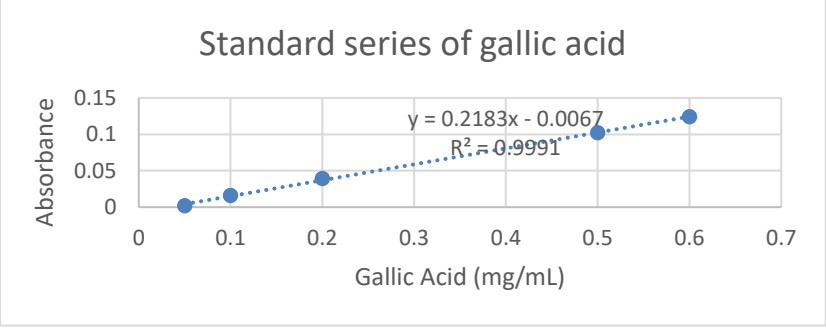

Figure 5. The curve of the standard gallic acid concentration

The data obtained showed that the phenols content of aqueous is higher than ethanol extract (19.2± 0.75, 9.16 ± 0.23) mg GAE/1g DE respectively, this is due to the difference in polarity of the two solutions. The polarity of the solution plays an essential role in the process of extracting phenols and liberating them from the plant material. The extraction is also influenced by the nature of the extracted phenolic extract. There are few studies on the fruits of the plant, as most of the research has focused on the leaves or used different sweeteners. However, a study

by Almutairi G [16] and colleagues confirms that the fruits have the lowest phenolic content.

3.3. The antioxidant activity

The antioxidant activity of *styrax* fruits is demonstrated in **figure 6**, where it shows the ability to scavenge 50% of free radicals (diphenylpicrylhydrazyl) and convert them into non-radical compounds (diphenylpicrylhydrazine). A linear relationship was also observed between the total phenolic content and antioxidant activity of *S. officinalis*. The antioxidant activity was expressed as IC_{50}, which is the amount of sample required to reduce the initial DPPH concentration by half.

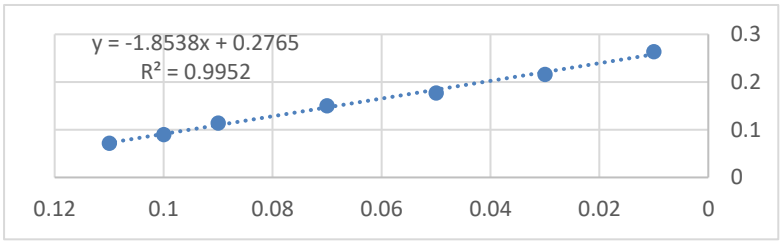

Figure 6. Free Radical Scavenging Activity of Gallic Acid

Table 4. Concentration of aqueous extract and percentage of DPPH inhibition

Concentration (mg/ml)	**0.5**	**1.25**	**2.5**	**3.75**	**6**
RSA% First iteration	46.48	47.89	56.34	64.08	77.46
RSA% Second repetition	49.3	51.05	59.86	67.25	79.23
RSA% Third repetition	47.53	49.65	58.45	66.2	78.52
Average RSA%±SD	47.77± 1.42	49.53 ± 1.58	58.22± 1.77	65.84± 1.61	78.40± 0.89

RSA: scavenging the free radicals

Table 5. Concentration of ethanol extract and percentage of DPPH inhibition

Concentration (mg/ml)	**1.25**	**1.5**	**2.5**	**3.75**	**6**
RSA% First iteration	30.63	33.8	48,94	63.38	89.79
RSA% Second repetition	35.21	34.86	51.06	65.14	91.55
RSA% Third repetition	34.51	37.68	51.41	66.9	90.49
Average RSA%±SD	33.45± 2.47	35.44± 2	50.47± 1.34	65.14± 1.76	90.61± 0.088

The graph illustrating the relationship between concentrations and IC% from the previous values is shown in **Figure7**

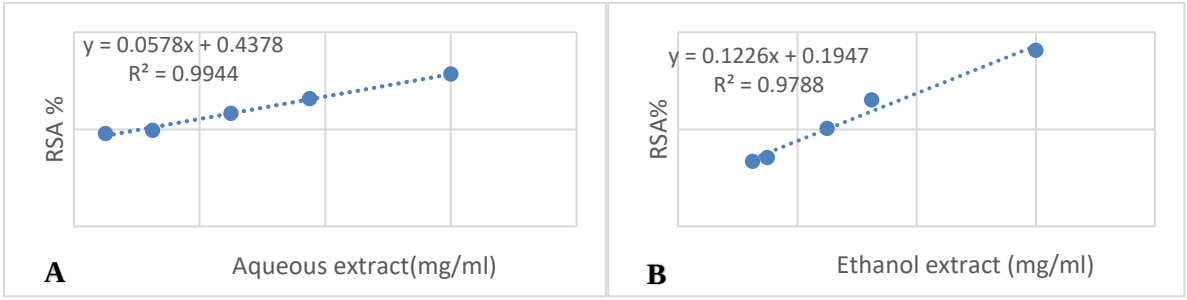

Figure 7. The linear relationship between concentration and IC (RSA) % measured at 517 nm for the aqueous extract (A) and the ethanol extract (B).

We obtained the IC_{50} values of Antioxidant activity from the linear equation for both extracts and the gallic acid standard, as shown in **Figure 8.**

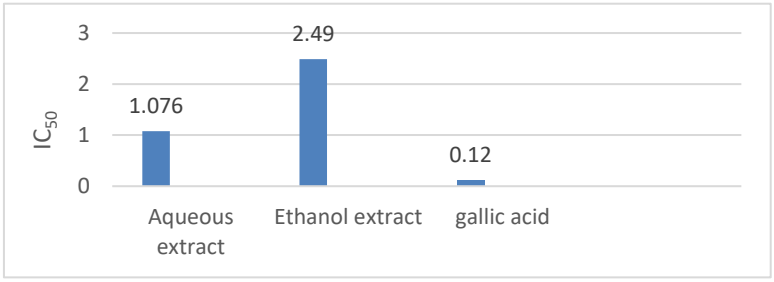

Figure 8. IC_{50} values of aqueous and ethanol extracts from S.officinalis pericarps and the gallic acid standard.

This study is consistent with the research conducted by silva et al [17] on the sensitization of Styrax camporum and S. ferrugineus fruits had weak free radical scavenging activity.

3.4. cholesterol-lowering activity

Table 6. Percentage reduction (IC%) of cholesterol for different concentrations of aqueous extract (three replicates).

Average IC%	IC%	Test absorbance	Aqueous extract concentration mg/ml	Standard absorbance
66.66 ± 2.7	64.42	0.053	2.5	0.149
	69.8	0.045		
	65.77	0.051		
61.29 ± 3.93	65.77	0.051	1.67	
	58.38	0.062		
	59.73	0.06		
55.35 ± 3.37	58.38	0.062	1.25	

Average IC%	IC%	Test absorbance	Aqueous extract concentration mg/ml	Standard absorbance
	55.7	0.066		
	51.68	0.072		
53.47 ± 1.69	51.68	0.072	1	
	55.03	0.067		
	53.69	0.069		
49.89 ± 3.03	46.98	0.079	0.5	
	49.66	0.075		
	53.02	0.07		

The graph of the previous data is used to derive the linear equation and calculate the IC_{50} (Figure 9).

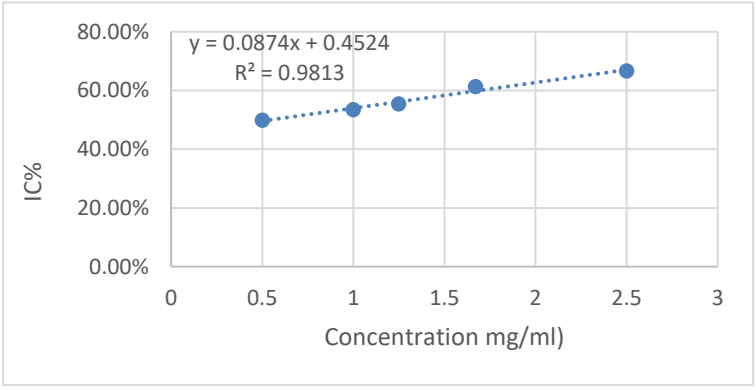

Figure 9. Significant correlation between different concentrations of aqueous extract and the percentage of cholesterol.

Table 7. Percentage reduction (IC%) of cholesterol for different concentrations of ethanolic extract (three replicates).

Average IC%	IC%	Sample absorbance	Ethanol extract concentration mg/ml	Standard absorbance
68 ± 3.38	71.14	0.043	16	0.149
	64.42	0.053		
	68.46	0.047		
58.61 ± 1.68	57.05	0.059	12	
	58.38	0.062		
	60.40	0.064		
51.9 ± 1.69	53.69	0.074	8	
	51.67	0.072		
	50.33	0.069		
47.2 ± 3.02	50.33	0.083	6	
	46.98	0.079		

Average IC%	IC%	Sample absorbance	Ethanol extract concentration mg/ml	Standard absorbance
	44.29	0.074		
44.74 ± 2.36	42.28	0.086	2	
	44.96	0.082		
	42.28	0.079		

The graph of the previous data is used to derive the linear equation and calculate the IC_{50} (**Figure 10**).

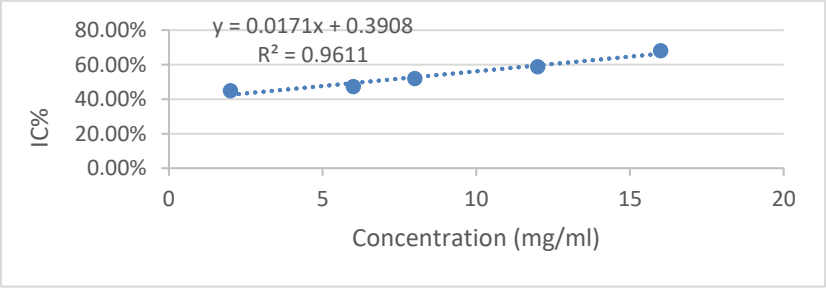

Figure 10. Significant correlation between the ethanolic concentrations of *S.officinalis* and reduction in cholesterol percentage.

Table 8. The IC_{50} values calculated from the linear correlation for both extracts.

IC_{50}	Extracts
0.54 mg/ml	Aqueous extract
6.4 mg/ml	Ethanolic extract

3.4. Statistical analysis

The results of the one-way ANOVA test (one-way analysis of variance) revealed significant differences in the phenol content between the ethanoic and aqueous extracts. The significance level for the mean differences was determined to be $P < 0.05$.

There is a significant correlation between the values of RSA% and IC% in the aqueous extract, where $p < 0.005$.

4. CONCLUSION

The study confirmed the presence of the sugar Styracitol in *S. officinalis* fruits [18], consistent with previous findings in related species [19] Additionally, the antibiotic compound Paromomycin was detected in both extracts. Notably, this study, for the first time, determined the chemical composition of *S. officinalis* fruits using GC-MS and found no toxic compounds.

Furthermore, the study revealed that the aqueous extracts of *S. officinalis* fruits exhibited higher antioxidant activity compared to the ethanolic extract, with IC50 values of 1.076 mg/ml and 2.49 mg/ml, respectively. The phenolic content in the two extracts was directly related to the IC50 value, with values of 19.2 mg GAE/1g DE and 9.16 mg GAE/1g DE for aqueous and ethanolic extracts, respectively.

It is important to note that there is a lack of reference studies on the fruits of *S. officinalis*. Most studies have focused on leaves or used different solvents compared to those used in the current study [8]. One study indicated that the fruits are considered to have the lowest phenolic content compared to other parts of the plant [16]. This study is the first to investigate the cholesterol-lowering

effectiveness of *S. officinalis* fruits. The study also highlighted the potential of *S. officinalis* as a valuable natural antioxidant resource.

The study also identified a linear relationship between extract concentrations and their efficacy in reducing cholesterol levels. Notably, the IC50 values for anti-cholesterol activity were 0.54 mg/ml for the aqueous extract and 6.4 mg/ml for the ethanolic extract, indicating that the aqueous extract was more effective at lowering cholesterol due to its superior ability to extract saponins and higher phenolic content [5,6]. As in many previous studies, there is a direct relationship between the phenolic content in extracts and their antioxidant and cholesterol-lowering abilities [20,21]. In conclusion, while this study provides valuable insights, further in vivo studies are recommended to understand the exact mechanism of cholesterol reduction and to isolate the active substances responsible for this effect.

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Conflict of Interest*:* The authors declare that there are no conflicts of interest.

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المحتوى الفينولي والفعالية الكاسحة للجذور الحرة والخافضة للكولستيرول لنبات الاصطرك الطبي المنتشر في الساحل السوري

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ملخص

الهدف من الدراسة: تحليل التركيب الكيميائي لثمار نبات الإصطرك الطبي *Styrax officinalis* باستخدام جهاز (GC-MS)، مع تحديد محتواها الكلي من الفينولات، وقدرتها الكاسحة للجذور الحرة، وفعاليتها الخافضة للكوليسترول.

الأساليب: طُبق المنهج التجريبي عبر تحديد المحتوى الفينولي الكلي بطريقة فولين–سيوكالتو (باستخدام حمض الغاليك كمعيار)، وقياس القدرة المضادة للأكسدة عبر كسح جذر (DPPH) الحر، بينما دُرست الفعالية الخافضة للكوليسترول لأول مرة بالطريقة الأنزيمية (CHOD/PAP method).

النتائج: كشفت النتائج عن وجود مركبات متنوعة، كان الـ(Styracitol) المركب الأساسي في المستخلصين المائي والإيثانولي.، تفوق المحتوى الفينولي في المستخلص المائي (19.2 ± 0.75 مغ/غ) على نظيره الإيثانولي (9.16 ± 0.23 مغ/غ) ، وفعالية مضادة للأكسدة بمتوسط قيم IC_{50} بلغت 1.076 مغ/مل (المائي) مقابل 2.49 مغ/مل (الإيثانولي) إضافة إلى تفوق ملحوظ للمستخلص المائي في خفض الكوليسترول (IC_{50} = 0.54 مغ/مل) مقارنة بالإيثانولي (IC_{50} = 6.4 مغ/مل).

الاستنتاجات: يُستنتج أن ثمار الإصطرك مصدر واعد للمركبات الفينولية ذات الخصائص المضادة للأكسدة والخافضة للكوليسترول، مما يدعم توظيفها في التطبيقات الطبية.

الكلمات الدالة: الفينولات الكلية، الإصطرك الطبي، فولين–سيوكالتو، مضادات الأكسدة، خافضات الكوليسترول

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Therapeutic Potential of Traditional Medicinal Plants from the Central Algerian Steppe for Treating Common Digestive Disorders

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ABSTRACT

In the central Algerian steppe, digestive system disorders are a common affliction that the majority of the population treats using medicinal plants. This study aims to evaluate the therapeutic potential of the medicinal plants used for treating four common disorders: colopathy, gastric ulcers, acute diarrhea, and chronic constipation. To achieve this, a survey was conducted using a semi-structured questionnaire, involving a total of 75 traditional phytotherapy practitioners. The questionnaire comprised two sections: socio-demographic information about the practitioners and details regarding the cited plants. Data were collected through field survey forms, categorizing participants by gender, age, education level, and professional experience. Detailed botanical information about the identified plants was carefully collected and analyzed. The diversity of medicinal plants used for digestive disorders was inventoried and assessed using adequate statistics. The survey identified 57 plant species across 32 botanical families, with a predominance of Lamiaceae (9 species) and Asteraceae (7 species). Infusion was the most common preparation method (44%) followed by decoction (38%). These findings emphasize the importance of medicinal plants in traditional treatment of gastric disorders in the region and provide a foundation for future studies on their biological and chemical potentials. The study identified a wide variety of medicinal plants used to treat conditions such as colopathy, gastric ulcers, acute diarrhea, and chronic constipation, with key plants like *Cuminum cyminum*, *Teucrium polium*, *Artemisia campestris*, and *Senna alexandrina* noted for their high efficacy. The use of single plants (8 species) for multiple disorders was observed, reflecting the interconnected nature of these conditions and the broad medicinal properties of the plants. This investigation underscores the extensive traditional knowledge and rich diversity of medicinal plants used in the central Algerian steppe for treating digestive disorders. The findings highlight the importance of these plants and suggest potential areas for further pharmacological research to validate their efficacy and safety.

Keywords: Therapeutic potential, digestive disorders, medicinal plants, central Algerian steppe, traditional medicine.

1. INTRODUCTION

The World Health Organization (WHO) estimates that the use of traditional medicine, particularly those based on medicinal plants, is on the rise, highlighting its critical role as a resource for the pharmaceutical industry. Between 20,000 and 25,000 plant species are utilized in traditional medicine, with over 50% of modern medications deriving from natural sources[1]. This widespread application underscores the essential role of medicinal plants in both traditional healthcare and the development of new

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pharmacological treatments. However, despite the increasing reliance on these plants, there remains a notable deficiency in research data within this field[2].

Due to its geographical position, the central Algerian steppe is noted for its significant floristic richness[3]. The flora of this region includes 170 taxa across 34 families and 111 genera. Dominant families in the region are Asteraceae, Poaceae, Fabaceae, Brassicaceae, and Cistaceae, which together account for approximately 59% of the species. The biogeographic spectrum reveals a predominance of Mediterranean species, with 80 taxa representing 47% of the recorded flora. Additionally, 27 endemic species, constituting 16% of the total, have been identified, with 16 of these being endemic to North Africa[4].

Digestive system disorders are common and have a substantial impact on global morbidity and mortality rates, being fifth leading cause of global mortality[5]. Individuals affected by these conditions are frequently diagnosed with colopathy, gastric ulcers, acute diarrhea, and chronic constipation[6,7]. These disorders represent one of the main reasons for medical consultation. The prevalence of this condition in the global population is estimated to be between 15 and 20%[8].Digestive system disorders are among the most frequently treated conditions with medicinal plants[9,10]. Considering the interconnected nature of these disorders, medicinal plants used for treatment often have multifunctional properties, addressing a range of digestive symptoms and conditions[8]. Despite this, most scientific organizations underestimate the significance of digestive system-related issues in health discussions[11].

In developing countries, medicinal plants remain a vital source of medication. Approximately 88% of people in these regions depends primarily on traditional medicine for their primary healthcare needs[12,13,14]. In the absence of a reference molecule, managing digestive system disorders presents significant challenges[15]. Given the limitations of current treatments, there was a strong need to explore new therapeutic approaches that are both effective and non-toxic. Medicinal plants offer a promising alternative, as they are generally more compatible with human physiology and tend to have fewer side effects. Additionally, given the significant economic impact and substantial social costs associated with digestive disorders[16], the collected data are crucial for identifying and researching new phytomolecules that could aid in the treatment and prevention of various digestive issues.

Therefore, this study aims to evaluatethe therapeutic potential of traditional medicinal plants used by the population of the central Algerian steppe for treating common digestive disorders, including colopathy, gastric ulcer, acute diarrhea, and chronic constipation.

2. MATERIALS AND METHODS

2.1. Study area

The study area was situated in the central Algerian steppe, situated in the central part of Algeria. This region serves as a transitional zone between the high steppic plains of the Tellian Atlas and the arid beginnings of the Saharian Atlas[17]. It spans between 2° and 5° east longitude and between 33° and 35° north latitude, covering an area of 32,280.41 km², which constitutes 1.36% of Algeria's total land area [3].The altitude varies from 1,613 meters in the east to 150 meters in the extreme south[18]. The number of inhabitants in the studied area was 1,491,370, served by approximately 260 phytotherapy practitioners. The inventory was conducted across 9 municipalities, gathering information from many traditional health practitioners based on their experiences. The investigation was performed in the following municipalities: Aïn Oussara, Had-Sahary, Hassi Bahbah, Zaafrane, Dar Chioukh, Djelfa, Charef, Faidh El Botma, and Messaad, as illustrated in **Figure 1**.

2.2. Climate

The climate of the study area is characterized by a semi-arid steppe environment. Summers are hot and dry, while winters are cold, with temperatures occasionally dropping below freezing. Precipitation is scarce and

irregular, with most rainfall occurring between November and April, although the annual totals remain low. The region is also notable for its strong winds, particularly in the spring, which often exacerbate the dry conditions and lead to dust storms.

2.3. Data collection

To document the medicinal plants used for treating digestive disorders, a traditional plant use survey was conducted using a semi-structured questionnaire. The study targeted four specific digestive conditions: colopathy, gastric ulcers, acute diarrhea, and chronic constipation. Conducted over one month in March 2024, the survey involved a representative sample of 75 herbalists. The semi-structured questionnaire was designed to gather comprehensive information in two main areas. The first section focused on the demographic and professional background (socio-demographic) of the herbalists, including their sex, age, education level, and years of professional experience. The second section detailed the plant materials used, documenting the local names of the plants, the specific parts utilized, the preparation methods, and the herbalists' perceptions of the plants' effects. This dual approach ensured a thorough understanding of both the practitioners' profiles and the traditional knowledge they possess.

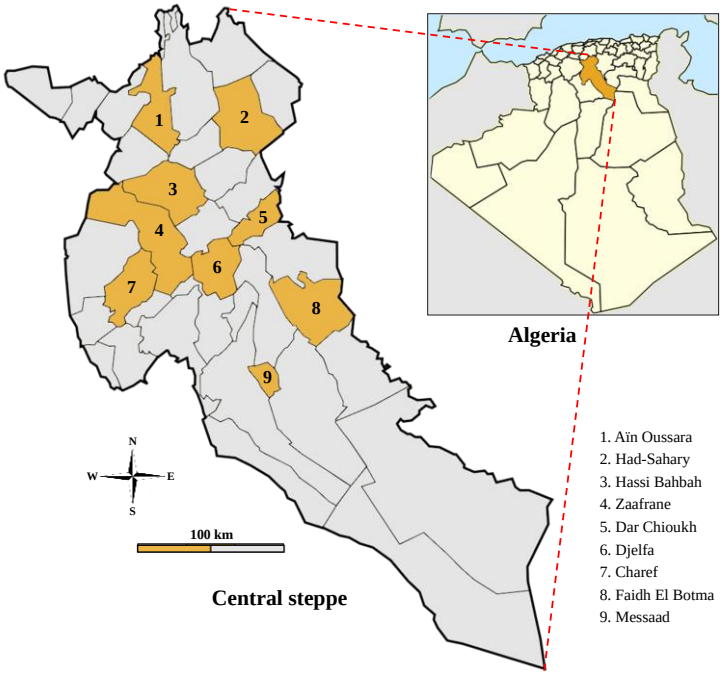

Figure 1. Geographical representation of the study area. The study sites are marked with a number corresponding to their geographic location.

The identification of plant species was conducted using several reference botanical sources on the vegetation and medicinal plants of the Algerian and North Africa, including the "Nouvelle Flore de l'Algérie" (New Flora of Algeria, the "Flore de l'Afrique du Nord" (Flora of North Africa), and the "Flore du Sahara" (Flora of the Sahara). The scientific name of each reported plant was verified using international botanical databases, such as The Plant List (https://www.theplantlist.org/) and Encyclopedia of Life (https://eol.org/).

2.4. Data analysis

The data collected from survey forms were manually entered into a database, processed, and analyzed using Microsoft Office 2016 Excel. Descriptive statistics, specifically percentages and frequencies, were used to evaluate the data. The Relative Frequency of Citation (RFC) was calculated as a percentage to assess the importance of each species for treating digestive disorders. The RFC was determined according to this formula: RFC = FC/N ($0<RFC<1$). FC was the number of informants citing the given species for a considered digestive disorder and N was the total number of informants for the same digestive disorder[19].The Family Importance Value (FIV) emphasizes the relevance of medicinal plant families. It was determined by dividing the relative frequency of citations for a particular family (RFC_{family}) by the total number of species belonging to that family (Ns). FIV = RFC_{Family}/Ns ($0<FIV\leq1$).

2.5. Data processing

The analysis of collected data involved various methods, such as the Number of Citations and the Relative Frequency of Citation and Family Importance Value. Data analysis employed simple descriptive statistical methods using Microsoft Office 2016 Excel. Quantitative variables were described using means, while qualitative variables were presented as frequencies and percentages. Graphical presentations were performed using OriginPro 2024 SR1 and Microsoft Office 2016 Excel. The statistical analysis was performed by XLSTAT (version 2018.1) and the level of significance was set at $p<0.05$.

3. RESULTS AND DISCUSSION

The general analysis of the results from this study showed that the use of plants in traditional medicine for treatingdigestive diseases was common among the population in the studied region.

3.1. Socio-demography of phytotherapy practitioners

A total of 75 phytotherapy practitioners were interviewed, representing approximately 28.8% of the 260 practitioners in the study area. This sample ensures a broad and diverse range of practices, experience levels, and regional representation. The participants, totaling 75, were categorized based on four socio-demographic characteristics: gender, age, education level, and professional experience, in accordance with the criteria defined byUmair *et al.*[20]. These characteristics were significant factors influencing the transmission of knowledge regarding the therapeutic uses of medicinal plants[21].

Regarding gender, 96% of the phytotherapy practitioners were men and 4% were women (**Figure2**), indicating a male predominance in this field. This observation was consistent with the findings of some other researches[22,23,24]. These results differ notably from those reported by Orch *et al.*[25], where the majority of phytotherapy users were women. This gender disparity could be attributed to various factors such as culture and traditions. The age distribution reveals that the majority of phytotherapy practitioners (56.19%) was in the 40 to 60-year age range. This was followed by those aged 20 to 40, who constitute 37% of the practitioners. Those aged over 60 and those 20 years or younger represent a smaller proportion of the total (**Figure2**). These results indicate a particular interest in phytotherapy among middle-aged individuals. Similar findings have been observed in other studies conducted worldwide[21,26,27].

Among phytotherapy practitioners, the highest proportion have a secondary school education (54.67%), followed by those with a middle school education (22.67%). Practitioners with a university-level education, or primary education, and those who were illiterate represent smaller proportions (**Figure2**). This study reveals that interest in traditional medicine spans all educational levels within the population. These results contrast with several studies that have shown that traditional knowledge in phytotherapy was primarily spread among individuals with low educational levels[28,29].

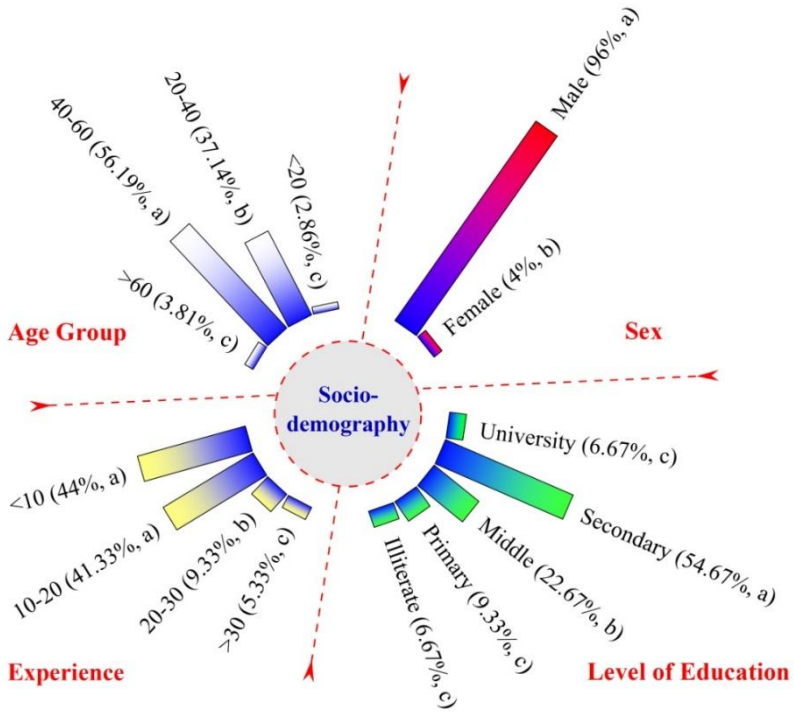

Figure 2. Socio-demographic characteristics of practitioners of traditional herbal medicine interviewed in the central Algerian steppe. ***For each socio-demographic parameter, percentage values with different letters indicate statistically significant differences (p<0.05; a>b>c).***

The distribution of practitioners based on professional experience was shown in **Figure 2**. A significant proportion of phytotherapy practitioners have less than 10 years of experience (44.00%), indicating a recent surge of interest in the field. A similar proportion, 41.33%, have between 10 and 20 years of experience, reflecting a stable and committed presence in the profession. In contrast, only 9.33% have between 20 and 30 years of experience, while just 5.33% have over 30 years, which may suggest a temporary decline in popularity or shifts in career focus. These results were comparable to those of Dey *et al.*[30], who found that a significant proportion of practitioners have less than 20 years of experience.

3.2. Diversity of medicinal plants used for the treatment of digestive disorders

The inventory of medicinal plants traditionally used in the treatment of digestive disorders in the central Algerian steppe includes a wide variety of species across different plant families. The **Table 1** categorizes these plants based on the specific digestive disorders, including colopathy, gastric ulcers, acute diarrhea, and chronic constipation.

The treatment of colopathy involves 23 species from 15 families such as Apiaceae, Lamiaceae, and Asteraceae. Notably, species like *Cuminum cyminum*, *Foeniculum vulgare*, and *Pimpinella anisum*from the Apiaceae family were commonly used, indicating a strong traditional preference for plants with carminative and anti-inflammatory properties. These plants were known to alleviate symptoms such as bloating, gas, and stomach cramps. The Asteraceae family was also well-represented, with *Matricaria chamomilla* being a commonly cited plant due to its calming and anti-inflammatory effects on the

digestive system. Studies have shown that the carminative effects of Apiaceae species were supported by their high essential oil content, which has been proven to relieve gastrointestinal discomfort[31]. Similarly, *Matricaria chamomilla*'s efficacy was attributed to its flavonoid content, which provides significant anti-inflammatory benefits[32]. The frequent use of these families indicates a strong traditional preference for plants with soothing and digestive-enhancing properties.

A total of 12 families and 16 species were used in the treatment of gastric ulcers. The Lamiaceae family was notably represented in the treatment of gastric ulcers, with plants like *Teucrium polium* and *Origanum majorana* being commonly cited. These results were consistent with those of Brahmi *et al.*[33], who indicated that digestive disorders are the primary therapeutic indications for plants in the Lamiaceae family. These plants were valued for their anti-inflammatory and protective effects on the gastric mucosa[34,35]. The Cupressaceae family, including *Juniperus phoenicea* and *Juniperus oxycedrus*, was also important in this category. These species were traditionally used for their antiseptic and anti-inflammatory properties, which help manage ulcer symptoms. The inclusion of *Punica granatum* from the Punicaceae family highlights the use of antioxidant-rich plants in preventing and treating ulcers[28].

For acute diarrhea, the inventory lists 10 families and 12 species. The Asteraceae family features prominently in the treatment of this disorder, with plants like *Artemisia campestris* being frequently mentioned. This family was known for its antimicrobial and anti-inflammatory properties, which were beneficial in managing diarrhea[36]. The Cupressaceae family also appears in this category, with species such as *Juniperus phoenicea* being used for their astringent and antimicrobial effects[37]. Additionally, *Zingiber officinale* from the Zingiberaceae family was highlighted for its digestive benefits and anti-inflammatory properties[38]. The reliance on these families underscores the importance of plants with antimicrobial and soothing effects in treating acute gastrointestinal issues.

The treatment of chronic constipation involves 8 families and 11 species. The plants from the Fabaceae and Euphorbiaceae families were particularly significant. *Senna alexandrina* from the Fabaceae family was a key plant known for its potent laxative properties[39], widely used to stimulate bowel movements[40]. *Ricinus communis* from the Euphorbiaceae family was another frequently cited plant, commonly used for its strong purgative effects[41]. The Apiaceae family, represented by *Foeniculum vulgare*, also plays a role in this constipation, valued for its mild laxative and carminative properties[42]. The presence of these families highlights the diverse botanical resources utilized in traditional medicine to manage chronic constipation through natural laxatives and digestive aids.

Table 1. Inventory of medicinal plants traditionally used in the treatment of digestive disorders in the central Algerian steppe

Family	Scientific name	Common name	Plant origin	Part used	Method of use	FC	RFC
Colopathy							
Aloaceae	*Aloe vera* L.	Aloe Vera	Spontaneous	Leaves	Mac/Inf/Dec	14	0.187
Anacardiaceae	*Pistacia lentis*us L.	Mastic Tree	Spontaneous	Fruits	Mac/Inf/Dec	68	0.907
Apiaceae	*Cuminum cyminum* L.	Cumin	Cultivated	Seeds	Mac/Inf/Dec	63	0.840
Apiaceae	*Pimpinellaanisum* L.	Anise	Cultivated	Seeds	Mac/Inf/Dec	53	0.707
Apiaceae	*Foeniculum vulgare* Mill.	Fennel	Cultivated	Roots	Mac/Inf/Dec	29	0.387
Apiaceae	*Thapsia garganica* L.	Deadly Carrot	Spontaneous	Leaves	Mac/Inf/Dec	19	0.253
Apiaceae	*Carum carvi* L.	Caraway	Cultivated	Seeds	Mac/Inf/Dec	16	0.213

Family	Scientific name	Common name	Plant origin	Part used	Method of use	FC	RFC
Asteraceae	*Matricaria chamomilla* L.	Chamomile	Spontaneous	Flowers	Mac/Inf/Dec	51	0.680
Asteraceae	*Cotula cinerea* Del.	Cotula	Spontaneous	Leaves	Mac/Inf/Dec	26	0.347
Fabaceae	*Trigonella foenum-graecum* L.	Fenugreek	Cultivated	Seeds	Mac/Inf/Dec	56	0.747
Lamiaceae	*Salvia officinalis* L.	Sage	Spontaneous	Leaves	Mac/Inf/Dec	59	0.787
Lamiaceae	*Mentha spicata* L.	Spearmint	Cultivated	Aerial part	Mac/Inf/Dec	24	0.320
Lamiaceae	*Origanum vulgare* L.	Oregano	Spontaneous	Aerial part	Mac/Inf/Dec	21	0.280
Lamiaceae	*Lavandula angustifolia* Mill.	Lavender	Spontaneous	Aerial part	Mac/Inf/Dec	7	0.093
Lauraceae	*Cinnamomum camphora* L.	Camphor Tree	Imported	Leaves	Mac/Inf/Dec	8	0.107
Linaceae	*Linum usitatissimum* L.	Flax	Spontaneous	Seeds	Mac/Inf/Dec	11	0.147
Myrtaceae	*Myrtus communis* L.	Myrtle	Spontaneous	Leaves	Mac/Inf/Dec	13	0.173
Rosaceae	*Rosa × damascena* Mill.	Damask Rose	Cultivated	Flowers	Inf/Dec	15	0.200
Rubiaceae	*Rubia tinctorum* L.	Madder	Imported	Seeds	Inf/Dec	12	0.160
Rutaceae	*Ruta montana* L.	Mountain Rue	Spontaneous	Aerial part	Mac/Inf/Dec	9	0.120
Simaroubaceae	*Ailanthus altissima* Mill. Swingle	Tree of Heaven	Spontaneous	Flowers	Mac/Inf/Dec	31	0.413
Verbenaceae	*Aloysia citrodora* Palau.	Lemon Verbena	Cultivated	Leaves	Mac/Inf/Dec	41	0.547
Zingiberaceae	*Curcuma longa* L.	Turmeric	Imported	Roots	Mac/Inf/Dec	37	0.493
Gastric ulcer							
Aloaceae	*Aloe vera* L.	Aloe Vera	Spontaneous	Leaves	Mac/Inf/Dec	26	0.347
Asteraceae	*Achillea millefolium* L.	Yarrow	Spontaneous	Leaves	Mac/Inf/Dec	17	0.227
Cupressaceae	*Juniperus phoenicea* L.	Phoenician Juniper	Spontaneous	Aerial part	Mac/Inf/Dec	62	0.827
Cupressaceae	*Juniperus oxycedrus* L.	Prickly Juniper	Spontaneous	Leaves	Mac/Inf/Dec	22	0.293
Fabaceae	*Glycyrrhiza glabra* L.	Licorice	Cultivated	Tree bark	Mac/Inf/Dec	14	0.187
Lamiaceae	*Teucrium polium* L.	Felty Germander	Spontaneous	Aerial part	Mac/Inf/Dec	69	0.920
Lamiaceae	*Origanum majorana* L.	Marjoram	Spontaneous	Leaves	Mac/Inf/Dec	20	0.267
Lamiaceae	*Ziziphora hispanica* L.	Spanish Hyssop	Spontaneous	Aerial part	Mac/Inf/Dec	18	0.240
Liliaceae	*Asparagus officinalis* L.	Asparagus	Imported	Leaves	Mac/Inf/Dec	15	0.200
Liliaceae	*Origanum vulgare* L.	Oregano	Spontaneous	Aerial part	Mac/Inf/Dec	12	0.160
Myrtaceae	*Myrtus communis* L.	Myrtle	Spontaneous	Fruits	Mac/Inf/Dec	21	0.280
Oleaceae	*Olea europaea* L.	Olive	Cultivated	Leaves	Mac/Inf/Dec	37	0.493
Pinaceae	*Pinus pinaster* Aiton	Maritime Pine	Spontaneous	Tree bark	Mac/Inf/Dec	16	0.213
Punicaceae	*Punica granatum* L.	Pomegranate	Cultivated	Fruit bark	Mac/Inf/Dec	46	0.613
Rosaceae	*Rosa × damascena* Mill.	Damask Rose	Cultivated	Flowers	Mac/Inf/Dec	24	0.320
Violaceae	*Viola abyssinica* Steud.	Abyssinian Violet	Spontaneous	Flowers	Mac/Inf/Dec	13	0.173
Acute diarrhea							
Asteraceae	*Artemisia campestris* L.	Field Wormwood	Spontaneous	Aerial part	Mac/Inf/Dec	46	0.613
Asteraceae	*Achillea moschata* Wulfen	Musk Yarrow	Spontaneous	Leaves	Mac/Inf/Dec	21	0.280
Cupressaceae	*Juniperus phoenicea* L.	Phoenician Juniper	Spontaneous	Aerial part	Mac/Inf/Dec	29	0.387
Cupressaceae	*Juniperus oxycedrus* L.	Prickly Juniper	Spontaneous	Leaves	Mac/Inf/Dec	25	0.333

Family	Scientific name	Common name	Plant origin	Part used	Method of use	FC	RFC
Fabaceae	*Ceratonia siliqua* L.	Carob Tree	Spontaneous	Fruits	Mac/Inf/Dec	23	0.307
Fagaceae	*Quercus ilex* L.	Holm Oak	Spontaneous	Fruits	Mac/Inf/Dec	27	0.360
Lamiaceae	*Rosmarinus tournefortii* de Noe	Turnefort's Rosemary	Spontaneous	Aerial part	Mac/Inf/Dec	34	0.453
Lauraceae	*Cinnamomum verum* J.Presl	Ceylon Cinnamon	Imported	Tree bark	Mac/Inf/Dec	41	0.547
Plantaginaceae	*Plantago ovata* Forssk.	Psyllium	Spontaneous	Leaves	Mac/Inf/Dec	15	0.200
Rhamnaceae	*Ziziphus spina-christi* L. Desf.	Christ's Thorn Jujube	Spontaneous	Fruits	Mac/Inf/Dec	19	0.253
Rhamnaceae	*Ziziphus juju*ba Mill.	Jujube	Spontaneous	Fruits	Mac/Inf/Dec	17	0.227
Zingiberaceae	*Zingiber officinale* Roscoe	Ginger	Cultivated	Stem	Mac/Inf/Dec	37	0.493
Chronic constipation							
Apiaceae	*Foeniculum vulgare* Mill.	Fennel	Cultivated	Seeds	Mac/Inf/Dec	39	0.520
Asteraceae	*Aucklandia costus* Falc.	Saussurea Costus	Cultivated	Tree bark	Mac/Inf/Dec	11	0.147
Asteraceae	*Artemisia herba-alba* Asso	White Wormwood	Spontaneous	Leaves	Mac/Inf/Dec	32	0.427
Euphorbiaceae	*Ricinus communis* L.	Castor Bean	Spontaneous	Fruits	Mac/Inf/Dec	25	0.333
Euphorbiaceae	*Croton tiglium* L.	Purging Croton	Cultivated	Seeds	Mac/Inf/Dec	61	0.813
Fabaceae	*Senna alexandrina* Mill.	Alexandrian Senna	Imported	Leaves	Mac/Inf/Dec	28	0.373
Fabaceae	*Ceratonia siliqua* L.	Carob Tree	Spontaneous	Fruits	Mac/Inf/Dec	13	0.173
Gentianaceae	*Centaurium erythraea* Rafn	Common Centaury	Spontaneous	Leaves	Mac/Inf/Dec	7	0.093
Lamiaceae	*Salvia hispanica* L.	Chia	Spontaneous	Seeds	Mac/Inf/Dec	16	0.213
Lauraceae	*Laurus nobilis* L.	Bay Laurel	Spontaneous	Leaves	Mac/Inf/Dec	15	0.200
Liliaceae	*Linum usitatissimum* L.	Flax	Spontaneous	Seeds	Mac/Inf/Dec	21	0.280
Malvaceae	*Malva parviflora* L.	Cheeseweed	Spontaneous	Leaves	Mac/Inf/Dec	19	0.253
Polygonaceae	*Rheum palmatum* L.	Chinese Rhubarb	Imported	Leaves	Mac/Inf/Dec	30	0.400
Zygophyllaceae	*Zygophyllum cornutum* Coss	Horned Zygophyllum	Spontaneous	Leaves	Mac/Inf/Dec	9	0.120

Mac: Maceration; Inf: Infusion; Dec: Decoction; FC: Frequence of citation; RFC: Relative frequence of citation;Total number of phytotherapy practitioners (N) is 75.

This data underscores the rich diversity of plant families used in traditional Algerian medicine for digestive disorders. The cultural knowledge embedded in the use of these plants was not only vital for preserving traditional plant uses but also offers potential avenues for pharmacological research into natural remedies for gastrointestinal health.

The data shows that Spontaneous plants were the most prevalent, comprising 42 (64.62%) of the total entries, indicating a strong reliance on locally available or naturally occurring plants. Cultivated plants follow with 16 plants (24.62%), suggesting a notable but secondary use of plants intentionally grown. Imported plants make up the smallest proportion at 10.77% (7 plants), reflecting their minor role in managing the studied digestive disorder.

3.3. Family importance and frequency of medicinal plant species

The distribution of Family Importance Values highlights a diverse range of plant families utilized for medicinal purposes. The FIV analysis provides significant insights into the cultural relevance of these families **(Figure 3)**. The families with the highest FIV values, including Punicaceae (0.61), Apiaceae (0.55), and Verbenaceae (0.55), were shown to play a prominent role in traditional medicinal

practices. Conversely, families such as Gentianaceae (0.09) and Zygophyllaceae (0.12) have the lowest FIV values, suggesting they were less emphasized in medicinal applications or used less frequently. Families like Lamiaceae and Asteraceae show moderate importance, indicating that while some families dominate medicinal use, others contribute to a broader spectrum of traditional remedies, particularly for digestive disorders.

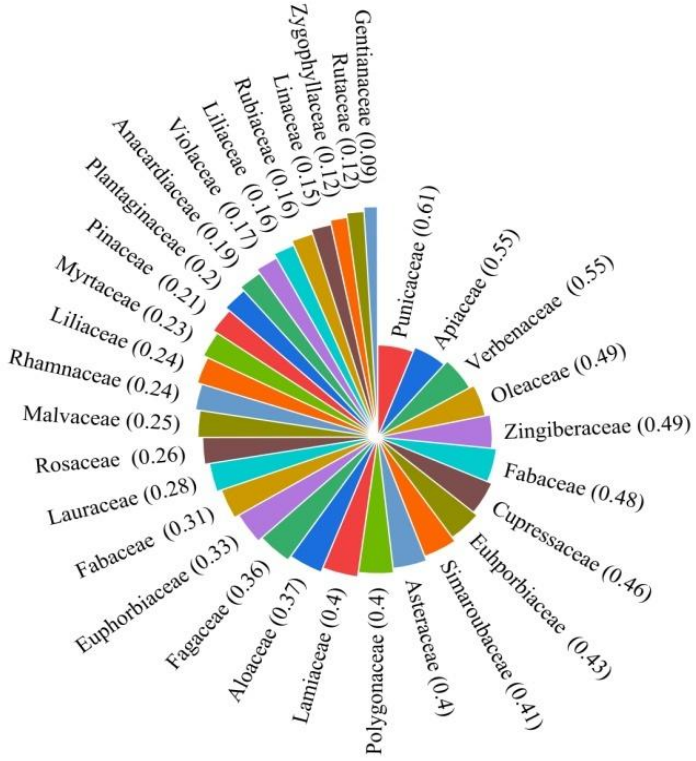

Figure 3. Importance of plant families used in treating digestive disorders in the central Algerian steppe.

The importance of plant families used for digestive disorders can vary significantly across traditional medicine surveys conducted in different regions[30,43]. This variation was due to differences in local knowledge and traditions, biodiversity, environmental conditions, and cultural practices. Each region has unique plant species and traditional uses influenced by local climate, soil, and cultural preferences, which affect the selection of plants for medicinal purposes. Additionally, factors such as plant availability, economic conditions, and the focus of ethnobotanical research further contribute to these variations[44].

The Relative Frequency of Citation, shown in **Figure 4**, underscores the significance of the plants used in local therapeutic practices and highlights the critical role of traditional medicine in treating digestive disorders.

The RFC for *Cuminum cyminum* was 0.91, highlighting its prominent use for colopathy. This high citation frequency was attributed to its well-known benefits for digestive health, primarily due to its carminative and anti-inflammatory properties[45]. *Pimpinella anisum* and *Salvia officinalis* were also frequently mentioned. *Pimpinella anisum* was particularly valued for relieving digestive bloating[46], while sage was recognized for its anti-inflammatory effects[47].

For gastric ulcers, *Teucrium polium* stands out with the highest RFC of 0.92, indicating its potential in protecting against ulcers through its anti-inflammatory and

antioxidant properties, which help reduce damage and promote healing of the gastric mucosa[48]. *Juniperus phoenicea* and *Punica granatum* were also notable, with contributions of 0.84 and 0.61, respectively. *Juniperus phoenicea* shows potential for antiulcer and antioxidant activity, suggesting it could be beneficial in protecting and healing the gastric mucosa[49]. *Punica granatum* was recognized for its anti-inflammatory, antioxidant, and mucosal protective properties, which may reduce damage and promote healing of the stomach lining[50].

In treating acute diarrhea, *Artemisia campestris* (RFC 0.61) and *Cinnamomum verum* (RFC 0.55) were frequently used. Research supports Artemisia's antimicrobial and antidiarrheal properties[51,52], while the benefits of cinnamon for digestive health, including diarrhea, were also supported[53]. For chronic constipation, *Senna alexandrina* was the leading plant with an RFC of 0.81, reflecting its potent laxative and purgative effects[54]. *Aucklandiacostus* (RFC 0.52) and *Ricinus communis* (RFC 0.40) also demonstrate significant effects.

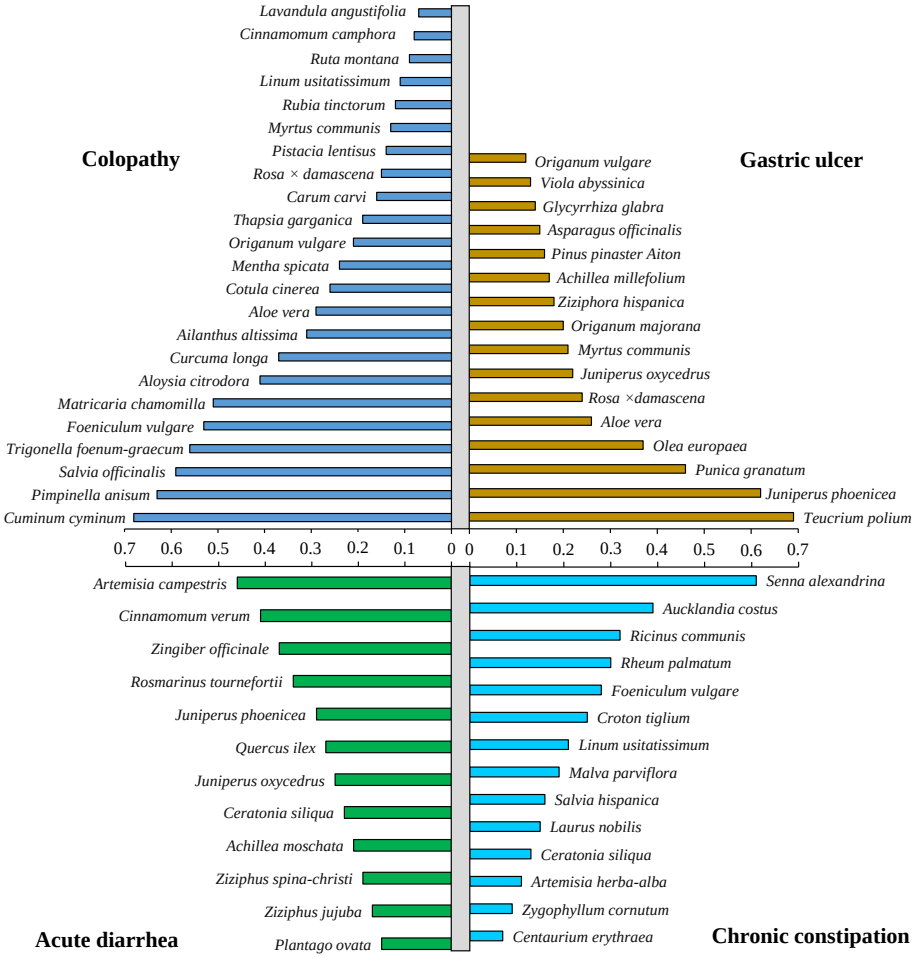

Figure4. Frequency of medicinal plant use in treating digestive disorders in the central Algerian steppe.

It has been observed that the use of a single plant for various disorders can be attributed to the interconnected nature of these conditions. When one digestive issue occurs, it was common for additional related disorders to manifest simultaneously. Additionally, the same plant can treat other disorders, showcasing its broad medicinal properties.

3.4. Relationship between medicinal plant parts used and preparation methods

The traditional medicinal use of different plant parts for treating digestive disorders was explored, with results summarized in **Figure5**. The preparation methods, including infusion, decoction, and maceration, were examined in relation to the most commonly used plant parts across various digestive disorders.

In the treatment of colopathy, seeds were the most commonly used plant part, with infusion (15.08%) and decoction (15.23%) being the preferred methods, while maceration was less used. Leaves were also prominently used, with a preference for decoction (11.57%), followed by infusion (8.93%), and then maceration. For flowers, infusion was the primary method, while maceration and decoction were less common. The aerial part of plants was least used, with decoction and infusion being more preferred. The use of fruit bark, tree bark, and stems was not observed in the treatment of colopathy.

For gastric ulcer, the aerial part and leaves were the most utilized plant parts, with infusion being the most popular method at 20.14% and 15.97%, respectively. Decoction follows for both the aerial part and leaves, around 12%, while maceration was less common for these parts. Flowers were primarily used through infusion and decoction. The use of fruits and fruit bark was minimal, while roots, seeds, and stems were not used for this digestive disorder.

In treating acute diarrhea, the aerial part was frequently utilized, with infusion as the preferred method at 14.67%, followed closely by decoction at 13.47%, while maceration was rarely adopted. Fruits also show significant usage, with infusion being the most chosen method (14.67%), followed by decoction and maceration, which were less popular. Leaves were prepared using decoction (7.78%) more often than infusion (6.89%) and sometimes with maceration. The use of tree bark was less common, with decoction followed by infusion being more commonly used. Flowers, roots, seeds, and fruit bark were not used in this context.

For chronic constipation, leaves were the most cited plant part, with decoction infusion being the leading method (21.47%), followed respectively by decoction and maceration. Seeds were also frequently used, with the methods used in the following order: decoction, maceration, and infusion. Fruits were primarily prepared through infusion (8%), followed by maceration and decoction. The use of tree bark was minimal, with infusion and decoction being more common. Aerial parts, flowers, roots, fruit bark, and stems were not used in treating chronic constipation.

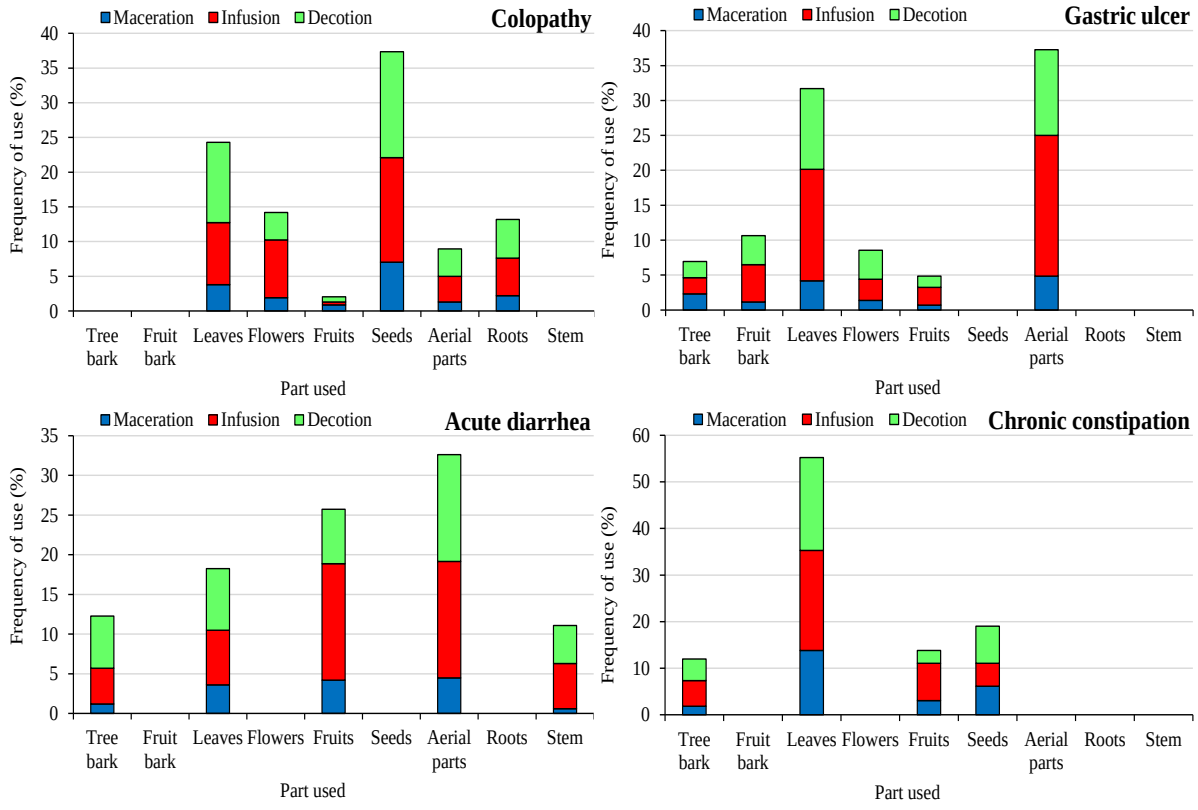

Figure5. Relationship between medicinal plant parts used and preparation methods for treating digestive disorders in the central Algerian steppe.

Overall, infusion followed by decoction were generally the most commonly employed methods across different plant parts for digestive disorders, particularly for the aerial parts and leaves. The choice of method often depends on the plant part and the specific disorder being treated, with decoction and maceration also being used frequently.

The parts of plants used in the treatment of digestive disorders play a crucial role in their therapeutic efficacy. Different parts of the plant, such as leaves, roots, fruits, and flowers, contain varying concentrations of bioactive compounds that target specific digestive issues. Leaves were commonly used for their high concentration of flavonoids, phenolic acids, and essential oils, which possess anti-inflammatory, carminative, and digestive-enhancing properties[55]. Similar compounds have been reported in leaves used for treating menstrual disorders, particularly in *Foeniculum vulgare* and *Trigonella foenum-graecum*[56]. Roots and rhizomes were valued for their high content of prebiotic compounds such as inulin, which promote healthy gut flora and improve overall digestive health[57]. Fruits were less commonly used but still play a role in the treatment of digestive disorders. Fruits were employed for their antioxidant properties, which help reduce oxidative stress in the digestive tract and protect against ulcers[58].

3.5. Efficacy of frequently cited plants in treating digestive disorders

The data detailing the efficacy of the most frequently cited plants in treating various digestive disorders provides significant insights, as shown in **Figure6**.

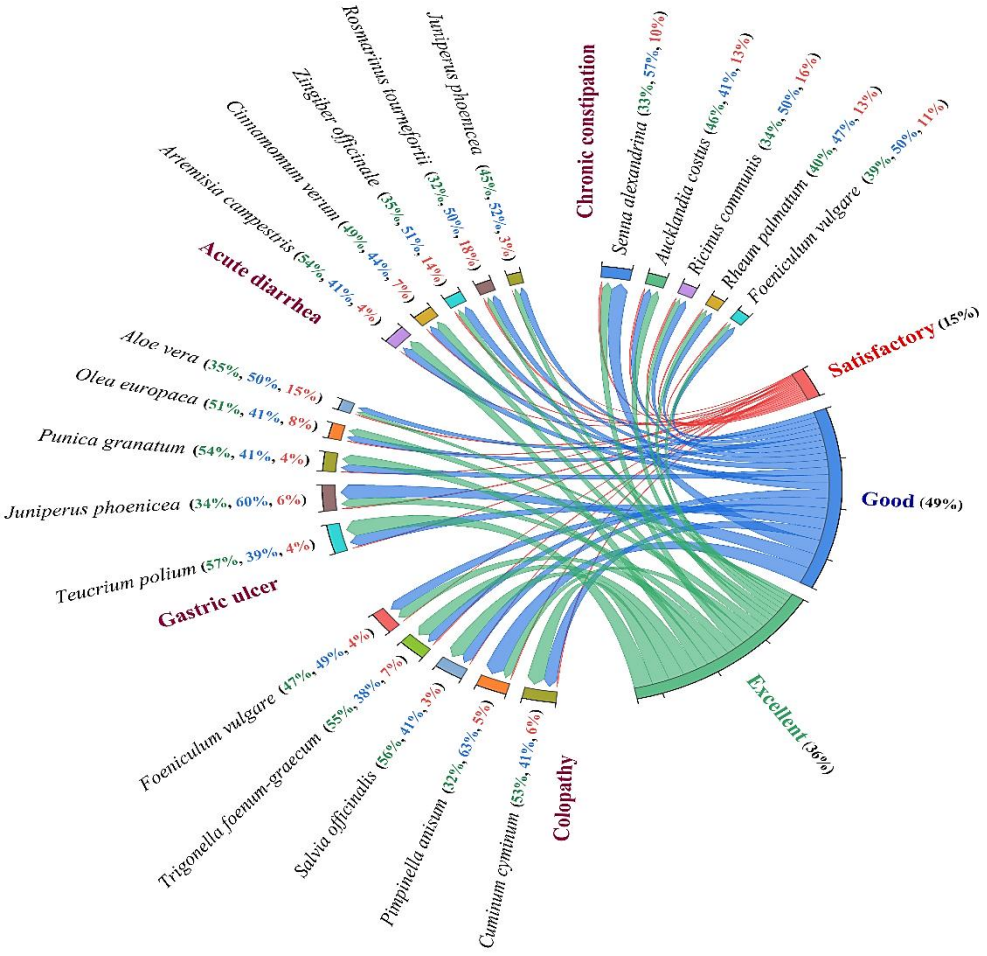

Figure6.Chord diagram showing the efficacy of the most frequently cited plants in treating digestive disorders in the central Algerian steppe. ***The values put in parenthesis for each plant represent the percentages of cited effects as satisfactory (Red), good (Bleu), and excellent (Green). The global citation percentages of the plants used are placed in parentheses next to each effect.***

For the treatment of colopathy, the most frequently cited plants with excellent effects were *Cuminum cyminum* (53%), *Salvia officinalis* (56%), and *Trigonella foenum-graecum* (55%). These plants were noted for their high efficacy, indicating that they were particularly effective in managing colopathy symptoms. On the other hand, *Pimpinella anisum* (63%) was primarily cited for its good effects, suggesting it was effective but not as potent as the others. *Foeniculum vulgare* shows a balanced efficacy, being equally cited for both good (49%) and excellent (47%) effects. Overall, the plants used for colopathy generally exhibit low frequencies of satisfactory effects, highlighting their strong therapeutic potential.

In treating gastric ulcers, *Teucrium polium* (57%) stands out with a high frequency of excellent effects, making it a key plant in traditional treatments. *Juniperus*

phoenicea also shows substantial effectiveness with a significant number of good (60%) effect. *Punica granatum* (54%) and *Olea europaea* (51%) were frequently cited for their excellent effects, indicating their role in traditional medicine for gastric ulcers. *Aloe vera* was less frequently cited for excellent effect but still plays a role in treatment. Generally, the plants used for gastric ulcers show high efficacy, with low frequencies of satisfactory effects.

For acute diarrhea, *Artemisia campestris* (54%) and *Cinnamomum verum* (49%) were frequently cited for their excellent effects, making them prominent in traditional treatments. *Zingiber officinale* and *Rosmarinus tournefortii* show moderate efficacy, with a balance of good and excellent ratings. *Juniperus phoenicea* was cited less frequently for excellent effects but still contributes to the treatment. The overall low frequency of satisfactory effects among these plants suggests their strong efficacy in managing acute diarrhea.

In the treatment of chronic constipation, *Senna alexandrina* was effective, with a significant number of good ratings (57%). *Aucklandiacostus* and *Ricinus communis* show considerable efficacy with excellent and good effects, with 46 and 50%, respectively. *Rheum palmatum* and *Foeniculum vulgare* exhibit a balanced efficacy, being cited for both good and excellent effects. The plants used for chronic constipation generally exhibit low frequencies of satisfactory effects, indicating their effectiveness in alleviating this condition.

The data highlights the significant role of various plants in traditional medicine for treating digestive disorders in the central Algerian steppe. Plants such as *Cuminum cyminum*, *Teucrium polium*, *Artemisia campestris*, and *Senna alexandrina* were particularly notable for their high efficacy across different digestive conditions. The low frequency of satisfactory effects among these plants indicates their strong therapeutic potential, emphasizing their value in traditional medicine.

The data illustrates that 49% of the citations reflect "good" effects, 36% indicate "excellent" effects, and 15% represent "satisfactory" effects. This distribution suggests that the majority of the cited plants exhibit substantial efficacy, with most evaluations falling between the "good" and "excellent" categories. These results highlight the strong reliance of the local population on traditional medicinal plants and the perceived effectiveness of these plants in the study region.

4. CONCLUSIONS

Our results reveal the richness of medicinal plant species in the central Algerian steppe for the traditional treatment of digestive disorders, highlighting the extensive traditional knowledge embedded in local practices. The socio-demographic analysis shows a predominance of middle-aged male practitioners with varying levels of education and professional experience, indicating widespread interest in phytotherapy across different social strata. The inventory of medicinal plants showcases a diverse range of species from multiple families, each valued for their therapeutic properties in treating conditions such as colopathy, gastric ulcers, acute diarrhea, and chronic constipation. Notably, plants like *Cuminum cyminum*, *Teucrium polium*, *Artemisia campestris*, and *Senna alexandrina* demonstrate high efficacy, underscoring their importance in traditional medicine. The frequent use of single plants for multiple disorders reflects the interconnected nature of these conditions and the broad medicinal properties of these plants. This study not only preserves valuable traditional practices but also suggests potential avenues for pharmacological research into natural remedies for digestive health. Further pharmacological studies should isolate and analyze active compounds in frequently cited plants to validate their efficacy and safety, while promoting conservation and sustainable use to preserve traditional knowledge and prevent resource depletion. The catalog of active plants could encourage specialized studies, potentially leading to the discovery of new products for modern therapeutics.

Acknowledgements

We extend our heartfelt thanks to the phytotherapy practitioners for their collaboration and for providing us with valuable information that greatly contributed to this work.

Ethical Consent

The information presented in this article is derived from a questionnaire-based survey conducted with phytotherapy practitioners. The authors did not perform any experimental studies involving humans or animals.

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الإمكانات العلاجية للنباتات الطبية التقليدية في منطقة السهوب الوسطى الجزائرية لعلاج الإضطرابات الهضمية الشائعة

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ملخص

في منطقة السهوب الوسطى الجزائرية، تُعد اضطرابات الجهاز الهضمي من الأمراض الشائعة التي يعالجها غالبية السكان باستخدام النباتات الطبية. تهدف هذه الدراسة إلى تقييم الإمكانات العلاجية للنباتات الطبية المستخدمة في علاج أربعة اضطرابات شائعة، وهي: التهاب القولون، القرحة المعدية، الإسهال الحاد والإمساك المزمن. ولتحقيق هذا الهدف، أُجري مسحٌ ميداني باستخدام استبيان، شمل 75 ممارسًا في مجال النباتات الطبية. تضمن الاستبيان قسمين: معلومات اجتماعية وديموغرافية عن المشاركين، وبيانات تفصيلية حول النباتات الطبية المُستخدمة. تم جمع البيانات من خلال استمارات المسح الميداني، مع تصنيف المشاركين حسب الجنس، العمر، المستوى التعليمي والخبرة المهنية. كما جُمعت معلومات نباتية دقيقة عن الأنواع المستعملة وتم تحليلها بعناية. وقد تم جرد وتقييم تنوع النباتات الطبية المستخدمة في علاج اضطرابات الجهاز الهضمي باستخدام أدوات إحصائية دقيقة. كشفت الدراسة عن 57 نوعاً نباتياً ينتمون إلى 32 عائلة نباتية، مع هيمنة واضحة لعائلتي الشفوية (Lamiaceae) بتسعة أنواع، والنجمية (Asteraceae) بسبعة أنواع. وكانت طريقة التحضير الأكثر شيوعاً هي النقيع (44%)، تليها الغلي (38%). تؤكد هذه النتائج الدور الهام للنباتات الطبية في العلاج التقليدي لاضطرابات الجهاز الهضمي في هذه المنطقة، كما توفر قاعدة علمية للبحوث المستقبلية حول الخصائص البيولوجية لهذه الأنواع. وقد حددت الدراسة مجموعة متنوعة من النباتات الطبية المستخدمة في علاج حالات مثل التهاب القولون، القرحة المعدية، الإسهال الحاد والإمساك المزمن، مع الإشارة إلى فعالية أنواع بارزة مثل الكمون (*Cuminum cyminum*)، الجعدة (*Teucrium polium*)، الشيح الصحراوي (*Artemisia campestris*)، والسنا (*Senna alexandrina*) نظرًا لفعاليتها العالية. كما لوحظ استخدام ثمانية أنواع نباتية لعلاج اضطرابات متعددة، مما يعكس الترابط بين هذه الحالات المرضية والتنوع الواسع في الخصائص العلاجية للنباتات المستخدمة. وتُبرز هذه الدراسة الثروة المعرفية التقليدية الواسعة والتنوع الغني للنباتات الطبية المستخدمة في منطقة السهوب الوسطى الجزائرية لعلاج الاضطرابات الهضمية. كما تُسلط الضوء على أهمية هذه النباتات وتُشير إلى مجالات واعدة لأبحاث دوائية مستقبلية تهدف إلى التحقق من فعاليتها وسلامتها.

الكلمات الدالة: الإمكانات العلاجية، الاضطرابات الهضمية، النباتات الطبية، السهوب الوسطى الجزائرية، الطب التقليدي.

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The Dispensing of Non-Prescribed Antibiotics to Pediatrics in Community Pharmacies: A Simulated Client Study

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ABSTRACT

Objective: This study aims to explore the practice of dispensing non-prescribe antibiotics to pediatrics in the community pharmacies in Jordan.

Method: This study was a cross-sectional study, performed between August 2021 and March 2022. Five different clinical case scenarios were simulated including pharyngitis, bronchitis, otitis media, gastroenteritis, and urinary tract infection (UTI). Three levels of demand were used to conceive the pharmacy staff to sell antibiotics.

Results: A total of 207 community pharmacies in Jordan were visited. The majority of pharmacies (n= 163, 78.7%) dispensed antibiotics without a prescription using three levels of demands. Most of the antibiotics dispensed for the pharyngitis case scenarios (95.3%), followed by UTI (89.2%). Among the pharmacists who dispensed antibiotics, 92.0% explained how to take the antibiotic, 41.1% provide the duration of treatment for the dispensed antibiotic, and 27.0% inquired about any type of drug allergy. On the other hand, only 21.3% (n= 44/207) of the pharmacy staff had refused to dispense any type of antibiotics, of those (n= 17/44, 38.6%) recommended consulting a physician, in which health issues were the only reason behind that refusal.

Conclusions: The results of the current study strongly demonstrate that dispensing of non-prescribed antibiotic in pediatric patients is prevalent in Jordan despite the current legislations. The ease of access and the inappropriate overuse of antibiotics confirms the need for stringent enforcement of the existing laws and the establishment of a new regulation regarding the dispensing of antibiotics without a valid prescription in the near future.

Keywords: Antibiotics; non-prescription; simulated client; pharmacists; Jordan.

1. INTRODUCTION

Antibiotics are potent medications designed to either kill bacteria or inhibit their growth [1]. They are among the most frequently prescribed medications in pediatric care and can significantly improve health outcomes in children when the bacteria are susceptible to the administered agent.[2]. Inappropriate use of these agents has led to the emergence of bacterial resistance, which is considered a challenging global health problem in recent years [3].Various studies have linked antibiotic misuse with negative outcomes that can be detrimental to the child, including atopic disease, obesity, irritable bowel syndrome, and a decrease in gut microbiota [4]. Antibiotic resistance is expected to cause more than ten million deaths per year by 2050 [5]. Therefore, immediate interventions must be developed and implemented to protect the child from the risk associated with improper antibacterial usage [6].

Dispensing antibiotics without a medical prescription

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(self-medication) in community pharmacies increases the inappropriate use of antibiotics, particularly in pediatric diseases since the majority of infections are caused by viruses that preclude the need for antibiotics except in some cases [7]. Despite legislation to the contrary, high rates of non-prescribed antibiotics have been observed in Jordan [8]. Several reasons can push this behavior, including a lack of awareness about laws and their prohibition, patient requests with insistence, and the fear of losing customers [7, 9].

Several studies have been performed to assess the practice of dispensing antibiotics without prescription among different populations [10-12]. Some studies were primarily concerned with the patient's perceptions, while others examined the practice from the perspective of the healthcare practitioner, whether in a hospital or a community pharmacy [13].

Previous studies conducted in Jordan found that antibiotics are still being dispensed as self-medication despite national legislation prohibiting this practice [14, 15] . There is limited evidence regarding the self-medication of antibiotics in the pediatric population in Jordan. Therefore, this study aims to evaluate the practice of dispensing non-prescribed antibiotics to pediatrics in community pharmacies in Jordan.

2. METHODS

2.1 Study design and sampling method

This was a cross-sectional simulated client's study that was conducted at different community pharmacies in different cities in Jordan, including Irbid, Amman, and Al-Mafraq, between August 2021 and March 2022. Each pharmacy was visited once by a single hidden investigator who pretended to have a relative pediatric patient with predetermined clinical case scenarios and pre-defined information. There were five predefined clinical case scenarios, but each pharmacy staff member encountered only one of these scenarios. Additional details were provided only in response to requests from pharmacy staff and were considered supplementary information. To be included in the study, pharmacy staff members (pharmacists or pharmacist assistants) had to be registered with the Jordanian Pharmacists Association (JPA). Pharmacists were selected using a convenience sampling method. Any staff member known to the investigator was excluded to enhance internal validity and avoid potential bias. Consequently, eight pharmacies were removed from the study: five in Irbid and three in Amman.

2.2 Clinical scenarios

Five different clinical case scenarios were chosen to be simulated in community pharmacies. These include pharyngitis (sore throat), bronchitis, otitis media, gastroenteritis, and urinary tract infections (UTI). Each scenario was assigned a number to facilitate dealing with the data: pharyngitis was assigned number one, bronchitis number two, otitis media number three, gastroenteritis number four, and urinary tract infection number five. Then these scenarios were distributed almost equally among pharmacies in the study sample. The pharyngitis (sore throat) scenario involved a 7-year-old girl suffering from a sore throat, pain while swallowing, and a fever for the previous 24 hours. While the bronchitis scenario included a 5-year-old boy suffering from a nonproductive cough and fever for the previous 24 hours. The otitis media scenario was for a 5-year-old boy suffering from ear pain and poor sleep for the previous 24 hours. The fourth scenario was gastroenteritis for a 12-month-old girl suffering from diarrhea and fever for the previous 24 hours. Finally, the UTI scenario was for a 7-year-old girl suffering from urgency, frequency, and dysuria for the previous 24 hours.

2.3 Levels of demand

To acquire antibiotics, the investigator followed three levels of demand. Level one of demand is where the investigator simulates the case scenario by asking for something to relieve the symptoms of the current disease. Level two of demand: when no antibiotic was given by the pharmacy staff, the investigator asked for a stronger medication. Level three of demand is a direct request for

antibiotics if the first two levels of demand fail. After leaving the pharmacy, the simulated patient filled out a standardized data collection form with prepared questions regarding the pharmacy. Following that, the antibiotic recommended by the pharmacist or pharmacist assistant was purchased and donated to the healthcare facility in collaboration with the Jordan University of Science and Technology (JUST). Medications other than antibiotics were not purchased due to fund limitations.

The data collection form was developed from previously published study that examined comparable research aims and objectives [14]. The data collection form contains three different parts. The first part consisted of the general information about the pharmacy staff and the pharmacy, including the gender, specialty, working shift, pharmacy area, pharmacy type, and its location. The second part included the clinical scenario's number and the decision of the pharmacy staff to administer antibiotics or not. The third part contains specific details about the predicted clinical case discussion between the pharmacy staff and the researcher. This part was further split into two sections, where the investigator might reroute the discussion and ultimately lead to one of two possible conclusions depending on the response. If the pharmacy staff dispensed an antibiotic, the investigator stated the level of demand (one, two, or three), the type of antibiotic given, if the pharmacy staff provided patient counseling, determined the duration of the treatment, inquired about the potential risk of drug allergies and other symptoms, the use of other medications at the same time, and whether the pharmacy staff recommended referral to a doctor.

The second section of the questionnaire contains information about the reason for the antibiotic dispensing refusal. The response of the pharmacy staff was graded as "administrative issues, health issues, or both." The investigator documented what kind of medicine was given instead of the antibiotic and if there was any advice to consult a doctor. Health issues were used for any reason concerning the health of the child; antibiotics cannot be administered for viral infections, and improper use of antibiotics can lead to the emergence of bacterial resistance. However, an administrative issue would only arise if the pharmacy staff refused to sell antibiotics because it was against the regulation or law (e.g., antibiotics can't be sold as OTC medications without a prescription).

The simulated investigators received extensive training from a senior clinical pharmacist to persuade the pharmacy staff that the clinical case described by the investigators was real. Before conducting the study, the investigators visited several community pharmacies that were excluded from the study. It aims to let the investigator be more flexible with data collection. To reduce the "Hawthorn effect," all visited pharmacies were not informed about the client method until the end of the visit. In all scenarios, the simulated clients pretended to be the child's older sister.

2.4 Ethical Approval

The present study received ethical approval from the Institutional Review Board (IRB) at the Jordan University of Science and Technology (JUST) (Reference number: 16/141/2021). The research protocol was also approved by the Jordanian Pharmacists Association. Deception and incomplete disclosure to study subjects (pharmacy staff) were considered ethically acceptable because this was a minimal-risk study, and it could not have been performed with complete disclosure of the investigator entity. All the collected information was kept anonymous.

2.5 Sample Size Determination

The sample size for this study was determined to ensure comparability with a previous study conducted in Jordan that had similar objectives but focused on adults [14]. Given that the earlier study recruited 202 participants, we aimed to recruit more than 200 participants in this study to maintain consistency and enhance the robustness of the findings.

2.6 Statistical Analysis

All the collected data were coded, entered, and analyzed using the Statistical Package for Social Sciences (SPSS) version 28.0. The descriptive analysis was conducted using

mean and standard deviation (SD) for continuous variables, while frequency and percentages were used for categorical variables. To evaluate the associations between categorical variables, a Pearson Chi-square test and a Fischer exact test were performed. An alpha level of 0.05 was used for all statistical tests and considered statistically significant. All tests were two-tailed.

3. RESULTS

3.1 Socio-Demographic Characteristics

A total of 207 pharmacies were visited from 3 different cities in Jordan; of those (n=97, 46.9%) were in Irbid, (n=87, 42%) were in Amman, and (n=23, 11.1%) were in Al-Mafraq. Five simulated-case scenarios were equally distributed among the pharmacies included in the study. In summary, pharmacists occupied 189 (91.3%) of the visited pharmacies, while pharmacist assistants occupied only 18 (8.7%). Among those recruited, 59.9% (n = 124) were females. Moreover, most of the visited pharmacies were independent (n = 170, 82.1%) and located on the main road (n = 141, 68.1%). For more details about the demographics, refer to Table 1.

Table 1. Socio-demographic characteristics

Variable	Frequency
Gender	
○ Female	124 (59.9)
○ Male	83 (40.1)
Specialty	
○ Pharmacist	189 (91.3)
○ Pharmacist assistant	18 (8.7)
Working shift	
○ Shift A	108 (52.2)
○ Shift B	99 (47.8)
Area of the pharmacy	
○ Irbid	97 (46.9)
○ Amman	87 (42.0)
○ Al-Mafraq	23 (11.1)
Pharmacy location	
○ Main road	141 (68.1)
○ Subside street	66 (31.9)
Pharmacy type	
○ Independent pharmacy	170 (82.1)
○ Chain pharmacy	37 (17.9)

3.2 Antibiotic Dispensing Practice

Antibiotics were dispensed without a medical prescription in 163 pharmacies (78.7%) with different levels of demand. Simulated cases of pharyngitis accounted for the highest percentage of antibiotic dispensing without a medical prescription (n=41, 95.3%), followed by UTI (n= 33, 89.2%), otitis media (n=36, 87.8%), gastroenteritis (n=32,74.4%), while the lowest percentage of antibiotic sales was for the bronchitis case scenario (n=21, 48.8%). The percentage of pharmacies that dispensed antibiotics without a prescription with different levels of demand for the simulated scenarios is summarized in Table 2.

Table 2. Dispensed non-prescribed antibiotics stratified by the level of demand (n= 207)

Level of Demand	Pharyngitis n=43	Bronchitis n=43	Otitis media n=41	Gastroenteritis n=43	UTI n=37	Total n=207	P-value#
Level One	39 (95.1)	9 (42.9)	24 (66.7)	27(84.4)	31 (93.9)	130 (79.8)	<0.001*
Level Two	2 (4.9)	6 (28.6)	8 (22.2)	4 (12.5)	1 (3)	21 (12.9)	
Level Three	0 (0)	6 (28.6)	4 (11.1)	1 (3.1)	1 (3)	12 (7.4)	
Total	41 (95.3)	21 (48.8)	36 (87.8)	32 (74.4)	33 (89.2)	163 (78.7)	

*UTI: Urinary tract infection, #using Fischer Exact test. * Significant at 0.05 significance level.*

Regarding the acquisition of antibiotics based on demand level, of all the pharmacies visited, the majority of antibiotics were dispensed directly after the clinical scenario (demand level one) (n=130, 79.8%); this was followed by level two of demand (n=21,12.9%) and finally level three of demand (n=12, 7.4%). as depicted in **Table 2**.

3.3 Pharmacy staff counseling following antibiotics dispensing

Most pharmacy staff in all clinical scenarios who dispensed antibiotics explained to the simulated patient how to take the antibiotic (n=150, 92%); pharmacy staff explained how to use antibiotics in relation to UTI (n= 33, 100%), bronchitis (n= 20, 95.2%), gastroenteritis (n= 29, 90.6%), pharyngitis (n= 37, 90.2%), and otitis media (n= 31, 86.1.%). The duration of treatment was specified by (n= 67, 41.1%) of the pharmacy staff for all clinical scenarios as the following: UTI (n= 19, 57.6%), bronchitis (n= 10, 47.6%), pharyngitis (n= 16, 39.0%), otitis media (n= 14, 38.9%), and gastroenteritis (n= 8, 25.0%).

Similarly, the majority of pharmacy professionals (n= 98, 60.1%) asked whether the child had symptoms other than those presented in the case simulation. Pharyngitis (n=23, 56.1%), bronchitis (n=13, 61.9%), otitis media (n=22, 61.1%), gastroenteritis (n=19, 59.4%), and UTI (n=21, 63.6%) all showed almost a similar rate. Conversely, the majority of pharmacy staff who dispensed antibiotics didn't inquire about the concomitant use of other drugs in the simulated case scenarios. Only a few numbers (n = 5, 3.1%) had asked about the other drug used by the child, and only 7 pharmacy staff (4.3%) recommended physical consultation for the simulated case scenario. More details are presented in Table 3.

Table 3. Pharmacists counseling following antibiotics dispensing (n= 163)

Simulated clinical scenario	Explain how to take the antibiotic	Give the duration of treatment	Asked about drug allergy	Asked about other symptoms	Asked about concomitant use of other drugs	Recommended consulting a physician
Pharyngitis n=41	37 (90.2)	16 (39.0)	16 (39.0)	23 (56.1)	2 (4.9)	0 (0)
Bronchitis n=21	20 (95.2)	10 (47.6)	6 (28.6)	13 (61.9)	0 (0)	0(0)
Otitis media n=36	31 (86.1)	14 (38.9)	11(30.6)	22 (61.1)	1 (2.8)	3(8.3)
Gastroenteritis n=32	29 (90.6)	8 (25.0)	2 (6.3)	19 (59.4)	2 (6.3)	1 (3.1)
UTI n=33	33 (100.0)	19 (57.6)	9 (27.3)	21 (63.6)	0 (0)	3 (9.1)

UTI: urinary tract infection. Percentages were calculated per case scenario type (per row).

3.4 The dispensed Antibiotics

The most frequently dispensed antibiotic group differs based on the simulated case scenario. Penicillin and penicillinase inhibitors were the most frequently dispensed antibiotics for pharyngitis (n= 18, 43.9%), and otitis media in simulation (n= 16, 44.4%). Antiprotozoal and macrolide drugs were commonly used to treat gastroenteritis (n= 23, 71.9%) and bronchitis (n= 9, 42.9%). However, the most frequently dispensed antibiotic in the UTI scenarios was third-generation cephalosporin (n= 16, 48.5%).

3.5 Antibiotic dispensing refusal

Only 21.3% of pharmacy staff (n= 44) refused to dispense any type of antibiotic despite using three levels of demand. Regarding each clinical scenario, the majority of refusal responses came from bronchitis cases (n=22, 51.2%), whereas the lowest refusal response was associated with the pharyngitis case scenario (n=2, 4.7%). Accordingly, the pharmacy staff who refused to dispense antibiotics offered the main reason for the refusal, which was exclusively (n=44, 100%) related to their concern regarding health issues that the pediatric patient could experience.

3.6 Physician consultation to visit a physician

Among the pharmacy staff who refused to dispense antibiotics, 17 (38.6%) recommended the simulator to visit the physician. For the pharyngitis cases, only one pharmacy staff refused to dispense antibiotics and recommended consulting a physician. While most cases of UTI (n = 3, 75%) and gastroenteritis (n = 8, 72.7%) in the refusal group recommended that the simulator visit the physician. More details are presented in Table 5.

Table 5. Physician consultation to visit a physician (n= 44)

	Pharyngitis n=2	**Bronchitis n=22**	**Otitis media n=5**	**Gastroenteritis n=11**	**UTI n=4**	**Total n=44**
Physician Consultation to visit a physician	1 (50.0%)	2 (9.1%)	3 (60.0%)	8 (72.7%)	3 (75.0%)	17 (38.6%)

4. DISCUSSION

The results of our study revealed that antibiotics could be easily acquired and dispensed in Jordan without a medical prescription. Approximately 78.7% of the community pharmacies dispensed antibiotics without a prescription using the different simulated case scenarios, despite the fact that the law prohibits this practice.

Furthermore, most of the simulated medical conditions in this study were caused by viral infections, and our results showed that most pharmacy staff were more likely to dispense antibiotics inappropriately and were unable to differentiate between bacterial and viral infections. The use of antibiotics for nonbacterial or viral pathogens will encourage the emergence of antibiotic resistance, which is one of the most serious consequences of this practice [16]. This finding is similar to that of a study done in Sana'a, the capital of Yemen, in which 73.3% of the community pharmacies dispensed antibiotics as over-the-counter medications [17, 18]. Moreover, our finding is comparable to that of another study done in Sri Lanka, which reported a high rate of selling antibiotics without a prescription (61%)[17].

According to the current study, the most antibiotics were dispensed in the pharyngitis clinical scenario (95.3%), followed by UTI (89.2%) and otitis media (87.8%). These findings are similar to those of a study conducted in Ethiopia, which also showed that antibiotics were primarily dispensed for pharyngitis and UTIs[19]. Additionally, the findings of a previous study conducted in Jordan, which assessed the percentage of pharmacies

dispensing antibiotics without a medical prescription, revealed comparable results. In that study, antibiotics were dispensed in 97.6% of simulated cases of pharyngitis and 83% of urinary tract infections (UTIs). This further highlights the prevalent practice of dispensing antibiotics without proper oversight for these conditions. [14].

In our study, antibiotics were administered without a prescription at three different levels of demand. Most antibiotics (87.2%) were dispensed without a prescription when the simulator asked for any medication to alleviate her symptoms (first level of demands). Besides, our finding is also comparable with the finding of the study done in Pakistan by Ahmad et al., where 80.3% of antibiotics were dispensed without a prescription when the simulator asked for anything to relieve their symptoms (level one of demand) followed by levels 2 and 3, respectively [20].

In previous similar studies, the actor simulated the disease [20, 21]. While in our study, the actor simulated a sick sister whom the pharmacy staff did not actually meet. This fact alone should have stopped pharmacy staff from dispensing antibiotics without a prescription, but instead, we got offers of antibiotics at the same rate as the "sick actor simulation."

In our study, the majority of pharmacy staff (92%) explained to the simulated patient how to take the dispensed antibiotic. On the other hand, the duration of treatment was explained to the simulated patient in only 41.1% of the cases. A study done in Lebanon showed that 47.5% of pharmacists told the parent of the child the duration of the treatment. After presenting the predetermined clinical case scenarios, more than half of the pharmacists (60.1%) asked if the child had any other symptoms [22].

In addition, only a small percentage of pharmacy staff (3.1% and 27%, respectively) asked about the concurrent use of other medications or other drug allergies to reduce the risk of side effects. Allergy to antibiotics is an important aspect of childhood since the majority of children never undergo antibiotic allergy testing. Epidemiological studies have found that a penicillin allergy, particularly to amoxicillin, is prevalent in children [23]. In our study, penicillin was one of the most commonly dispensed antibiotic groups by community pharmacy staff.

Failure of the pharmacy staff to inquire about the concomitant use of other medications poses a significant risk. Antibiotics may interact with many medications if taken simultaneously. Therefore, the use of antibiotics in conjunction with other medications might result in significant drug-drug interactions that can either potentiate their effect or enhance their toxicity [24]. The results of our study did not match those of a study done in Ethiopia, where none of the pharmacists asked about allergy history or supplied information about possible drug interactions that could result in a high risk [19].

Amoxicillin/clavulanate was the most commonly prescribed antibiotic for presumed cases of otitis media and pharyngitis (44.4% and 43.9%, respectively). Likewise, in a study conducted in Saudi Arabia, penicillin/penicillinase inhibitor (amoxicillin/clavulanate) was the most dispensed antibiotic for pharyngitis and otitis media case simulation [24]. In the current study, macrolides (42.9%) were the most frequently dispensed antibiotic group in the bronchitis case simulation, while in China, cephalosporins (53.7%) were the most frequently dispensed antibiotics for the cough case simulation [25]. Antiprotozoals were dispensed in the majority of gastroenteritis case simulations (71.9%), whereas in India, antiprotozoals and fluoroquinolones were the most commonly dispensed antibiotics for acute gastroenteritis in children [26].

Our study showed that 21.3% (n = 44) of the pharmacy staff refused to dispense any type of antibiotic despite using the three levels of demand. The refusal to provide antibiotics for the simulated case scenarios was solely motivated by health concerns. The recommendation to consult a physician was not always followed; only 38.6%

(n = 17/44) of the pharmacy staff who refused to dispense antibiotics advised the simulated patient to see a doctor. Comparable results had been obtained from a study conducted in northeastern China, where the rationale behind refusing the dispensing of antibiotics without a valid prescription was solely related to health issues (Shi et al.). A study conducted in Italy discovered that 15.4% of pharmacists dispensed non-prescribed antibiotics because many patients couldn't afford a doctor's visit [27].

Antibiotic resistance has been reported in Jordan for most antibiotic classes, including penicillins, cephalosporins, monobactams, tetracyclines, aminoglycosides, fluoroquinolones, and sulfonamides [28]. Bacterial resistance has prompted governments and health regulatory agencies around the world to restrict antibiotic dispensing in community and hospital pharmacies [29]. In Jordan, non-prescription antibiotics are illegal, but regulatory agencies don't enforce the law [30]. Unfortunately, there are no national rules regulating the inappropriate use and distribution of antibiotics, which contributes to antibiotic resistance [30].

This study had several limitations. First, the small sample size may affect the generalizability of the findings. Additionally, the methodology involved applying three levels of demand until an antibiotic was dispensed, which resulted in the simulators receiving antibiotics despite the pharmacy professional's denial. This approach was intended to illustrate the impact of persistence in obtaining an antibiotic when it was not clearly necessary. However, this design choice could lead to biased results and does not accurately reflect real-world scenarios, potentially resulting in an overestimation of how frequently individuals might obtain antibiotics from dispensers. However, our study was directed mostly at pharmacies in three cities in Jordan, and one of these cities involved only a small number of pharmacies. Focusing on the north and the middle cities of Jordan may enhance the bias and not reflect the practice in other areas or countries, thus our findings may not necessarily be generalized to the entire country.

Despite this, we believe that the methodology of this study, using the simulation of clinical cases, is closer to the real-life scenario than methods employed in other studies, such as questionnaires, which are susceptible to misleading responses. In addition, our findings are robust and provide direction for the future.

5. CONCLUSION

The results of the current study strongly demonstrate that the dispensing of non-prescribed antibiotics to pediatric patients is prevalent in Jordan despite the current legislation. Accordingly, applicable interventions must be established and applied to protect the child from the impact associated with inappropriate antibacterial usage. Interventions should include the enforcement of the policy to prevent the over-the-counter sale of antibiotics.

6. Disclosure Statement

The Authors declare that there is no conflict of interest.

7. Funding statement

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صرف المضادات الحيوية بدون وصفة طبية للأطفال في الصيدليات المجتمعية: دراسة باستخدام العميل المتخفي

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ملخص

الهدف :تهدف هذه الدراسة إلى استكشاف ممارسة صرف المضادات الحيوية بدون وصفة طبية للأطفال في الصيدليات المجتمعية في الأردن.

المنهجية :كانت هذه الدراسة دراسة مقطعية أُجريت بين أغسطس 2021 ومارس 2022. تم تمثيل خمس حالات سريرية مختلفة شملت: التهاب البلعوم، التهاب الشعب الهوائية، التهاب الأذن الوسطى، التهاب المعدة والأمعاء، وعدوى المسالك البولية. تم استخدام ثلاث مستويات من الطلب لإقناع موظفي الصيدلية ببيع المضادات الحيوية.

النتائج :تم زيارة 207 صيدلية مجتمعية في الأردن. صرفت الغالبية العظمى من الصيدليات (عدد = 163، 78.7٪) المضادات الحيوية بدون وصفة طبية باستخدام مستويات الطلب الثلاثة. تم صرف معظم المضادات الحيوية لحالات التهاب البلعوم (95.3٪)، تلتها حالات عدوى المسالك البولية (89.2٪). من بين الصيادلة الذين صرفوا المضادات الحيوية، قام 92.0٪ بشرح كيفية تناول المضاد الحيوي، و41.1٪ ذكروا مدة العلاج، و27.0٪ سألوا عن وجود أي نوع من الحساسية للأدوية. من ناحية أخرى، رفض فقط 21.3٪ (44 من 207) من موظفي الصيدليات صرف أي نوع من المضادات الحيوية، ومن بين هؤلاء (17 من 44، 38.6٪) أوصوا بمراجعة الطبيب، وكان السبب الوحيد للرفض هو وجود مشكلات صحية.

الاستنتاج :توضح نتائج هذه الدراسة بوضوح أن صرف المضادات الحيوية بدون وصفة طبية للأطفال شائع في الأردن، على الرغم من التشريعات الحالية. تؤكد سهولة الوصول إلى المضادات الحيوية وسوء استخدامها على ضرورة التطبيق الصارم للقوانين الحالية وإنشاء تنظيم جديد بشأن صرف المضادات الحيوية بدون وصفة طبية في المستقبل القريب.

الكلمات الدالة: مضادات حيوية؛ بدون وصفة طبية؛ عميل متخفي؛ صيادلة؛ الأردن.

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Literature Review of Herbal Remedies Used for Diabetes Mellitus: Efficacy, Safety, and Regulatory Considerations

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ABSTRACT

The Problem: The rising prevalence of Type 2 Diabetes presents a significant global health burden and economic strain, driving interest in herbal medicine as a potentially cost-effective alternative or supplement to conventional treatments.

Experimental Approach: This literature review included peer-reviewed studies published between 2018 and 2024. A comprehensive search of databases such as PubMed, Scopus, and Google Scholar was conducted using terms related to diabetes, herbal medicine, and cost-effectiveness. The included studies originated from various countries, including India, China, the United States, the United Kingdom, Saudi Arabia, and several African nations. Studies targeting adult participants with diabetes and assessing the effectiveness, cost, or user experiences of herbal remedies were included. In contrast, studies involving children, non-peer-reviewed articles, and those not directly related to herbal medicine were excluded. Data extraction was performed independently by two researchers using a standardized form to collect information on study characteristics, sample size, herbal remedies, glycemic control outcomes, and side effects.

Major Findings: A total of 45 studies were included, comprising randomized controlled trials (RCTs) and observational studies. The findings indicated that specific herbal remedies can significantly enhance glycemic control, with an average reduction in HbA1c ranging from 0.5% to 1.5%. Additionally, the economic impact of these remedies showed potential cost advantages compared to conventional treatments, although their safety profiles revealed some associated side effects.

Conclusions: This review highlights the potential benefits of herbal remedies in managing Type 2 Diabetes but underscores the need for further research. Future studies should specifically address optimal dosages, interactions with conventional medications, cost-effectiveness, and long-term safety and efficacy, particularly through RCTs with larger sample sizes and extended follow-up periods.

Keywords: Herbal Medicine; Type 2 Diabetes; Cost-effectiveness; Glycemic Control; User profiles; Economic Impact.

1. INTRODUCTION

Type 2 Diabetes (T2D) is a chronic metabolic disorder characterized by elevated blood glucose levels, resulting from insulin resistance and impaired insulin secretion (1). The International Diabetes Federation estimated that approximately 537 million adults were affected globally in 2021, with projections reaching 643 million by 2030 and 783 million by 2045 (2). This escalating prevalence underscores the urgent need for effective management strategies.

While conventional management approaches offer some benefits, they often come with significant limitations, including side effects and high costs (3). These challenges have spurred interest in complementary and alternative

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medicine (CAM), particularly herbal remedies, as potential adjuncts or alternatives in T2D management (4).

Herbal medicine has a long history of treating various ailments, including diabetes, across many cultures. Recent scientific investigations have demonstrated how certain herbs may influence glucose metabolism. For instance, some herbal compounds may improve insulin sensitivity, stimulate insulin secretion, or inhibit carbohydrate-digesting enzymes (5).

Diverse bioactive compounds found in herbal medicine may help in the management of blood sugar levels (glycemic control). For example, polyphenols in green tea and berries demonstrate antioxidant properties that may protect pancreatic β-cells from oxidative stress-induced damage (6). Terpenoids in herbs such as bitter melon (*Momordica charantia*), have improved glucose uptake in muscle and adipose tissues (7). Notable herbal remedies include berberine from *Berberis vulgaris*, which has been shown to lower blood glucose levels (8). Similarly, cinnamon (*Cinnamomum verum*) has been studied for its ability to improve insulin sensitivity (9), while bitter melon is known for its hypoglycemic properties (10). Ginseng has also exhibited hypoglycemic effects in several studies (11).

However, the use of CAM products is not without risks, and safety concerns must be carefully considered. Drug-herb interactions pose significant challenges in the management of T2D. For instance, ginseng can interact with warfarin, potentially altering its anticoagulant effects (12). Bitter melon can also interact with insulin and oral antidiabetic medications, which may lead to dangerously low blood sugar levels (13,14). Furthermore, St. John's wort can significantly affect the metabolism of various drugs, including those for diabetes, potentially leading to decreased efficacy (13). The risk of hypoglycemia is a major concern with certain herbal remedies, particularly when used alongside conventional antidiabetic medications. This highlights the importance of careful monitoring and professional guidance when incorporating herbal remedies into diabetes management plans (13). Additionally, the lack of standardization in herbal products further complicates their safety profile. Herbal products may be contaminated with harmful substances like heavy metals or pesticides. For example, Fenugreek seeds have been found to contain contaminants that can lead to liver and kidney damage (15).

Integrating herbal medicine into mainstream T2D management faces several challenges. These include variability in herbal preparation methods, lack of standardization in dosing, potential drug-herb interactions, and limited understanding of long-term safety profiles. Moreover, the regulatory landscape for herbal products varies significantly across countries, complicating the assessment of quality and efficacy (16–18).

This review aims to provide a comprehensive overview of the current state of research on herbal remedies used for diabetes management. We will focus on the prevalence of use, cost considerations, and safety issues associated with these interventions while identifying critical research gaps, particularly regarding safety profiles, long-term efficacy, standardization, and effective communication between healthcare providers and patients.

While some studies have shown promise for certain herbal remedies in glycemic control, many lack rigorous scientific validation (19). Therefore, this review will evaluate the efficacy, safety, and economic implications of herbal remedies in T2D management.

2. METHODS

A comprehensive search of published peer-reviewed articles was conducted using the databases PubMed, Google Scholar, Scopus, ProQuest, CINAHL, and Science Direct. Search terms included "diabetes," "herbal medicine," "traditional medicine," "natural remedies," "botanicals" "complementary and alternative medicine," "patient satisfaction," "health beliefs," "economic burden," and “cost-effectiveness” and their synonyms. Boolean operators (e.g., AND, OR) were used to refine search

results. Additional studies were identified through hand-searching reference lists.

Inclusion and Exclusion Criteria

The inclusion criteria were peer-reviewed articles published from 2018 to 2024, focusing on adult participants with diabetes and assessing aspects related to the rate of herbal remedies use, cost, or socio-demographic characteristics of herbal remedy use. Only studies published in English were included. The included studies originated from various countries, including India, China, the United States, the United Kingdom, Saudi Arabia, and some African nations. Studies were excluded if they involved children or adolescents, were not peer-reviewed, or did not specifically address herbal medicine or diabetes. The included studies were primarily cross-sectional surveys, cohort studies, and RCTs.

Data Extraction

Data extraction involved information on study characteristics, sample size, types of herbal remedies used, the prevalence of herbal remedy use, costs, and socio-demographic factors of participants (including age, gender, income, and geographical location). A standardized data extraction form was utilized, and any inconsistencies were resolved through discussions among reviewers.

Methodological Rigor Assessment

Methodological rigor was assessed using the Cochrane Collaboration's Risk of Bias tool, evaluating aspects such as randomization, allocation concealment, blinding, and selective reporting. In our review, we included 45 studies, of which only 7 were assessed for risk of bias. The selection of these 7 studies was based on their methodological rigor and the applicability of the Cochrane Collaboration's Risk of Bias tool. While this tool is primarily designed for RCTs, it can also be applied to other study designs that exhibit sufficient methodological quality. The decision to assess only these 7 studies was made to ensure a focused evaluation of bias where the tool could be most effectively utilized. We acknowledge that the remaining studies were not assessed for bias, as they did not meet the criteria for this specific evaluation. This approach is intended to maintain the integrity of our review by concentrating on studies where a robust bias assessment was feasible.

Data Synthesis

While a meta-analysis was not conducted as part of this study, we reviewed several relevant meta-analyses in the literature. For instance, Alzahrani et al. conducted a systematic review and meta-analysis that explored the global prevalence and types of CAM use among adults with diabetes (20). Our overall findings are based on a variety of study designs, including RCTs, observational studies, and cross-sectional analyses. The goal of this narrative synthesis is to summarize the key findings and identify potential areas for future research.

3. RESULTS

Table 1 provides a comprehensive summary of various studies evaluating the effectiveness of herbal medicines for glycemic control in diabetes. It includes data from RCTs, observational studies, and cross-sectional analyses, highlighting different herbal interventions and their outcomes. For instance, Ahmad et al. conducted an RCT with 52 participants, finding significant reductions in fasting plasma glucose (FPG) and HbA1c levels with fenugreek and cinnamon, though insulin levels fell significantly only in the cinnamon group (21). Kandhare et al. reported that while fenugreek (IND-2) did not significantly reduce fasting blood sugar (FBS), there was noted improvement in HbA1c levels among 30 participants, with mild adverse events and a few discontinuations (22). Maideen reviewed a range of studies including a pilot study with 41 participants and a double-blind RCT with 250 participants, demonstrating significant improvements in fasting blood FBS and HbA1c levels with *Nigella sativa* (Black Seed) oil, showing comparable effectiveness to metformin in some cases (23). Prasopthum et al. in a cross-sectional study of 739

participants found that herbal medicines like bitter gourd, pandan leaf, and country mallow used in combination with antidiabetic agents, particularly bitter gourd, were associated with improved glycemic control (24). Chan et al. conducted RCTs on GanopolyTM, G. lucidum, and Lingzhi, reporting mixed results with GanopolyTM showing reduced fasting and postprandial glucose levels and HbA1c, while the other interventions showed no significant effects (25). Adam et al. found marked improvements in glycemic control and lipid profile with *Nigella sativa* oil, supported by in vivo studies demonstrating antidiabetic effects in rats (26). Yedjou et al. highlighted positive effects of garlic, bitter melon, *Hibiscus sabdariffa*, and ginger on blood glucose levels, with notable reductions observed across the board (5).

While detailed cost information was not consistently reported across studies, systematic reviews have highlighted the potential of herbal remedies; however, comprehensive cost-effectiveness analyses specifically focusing on these interventions are still lacking (27,28). This review acknowledges the inconsistencies in cost reporting and aims to provide a clearer analysis of cost considerations in future work. Overall, the findings indicate that certain herbal remedies may be effective in managing glycemic levels, but uncertainties regarding their cost-effectiveness remain. More rigorous cost analyses are necessary to fully understand their economic implications in diabetes management.

The safety profiles for herbal remedies were generally favorable, with only minor adverse effects reported in a few instances (5,23,24). For example, in the studies reviewed, most participants experienced mild adverse events with fenugreek and IND-2, while no major adverse effects were noted for *Nigella sativa*, which reported long-term safety during trials (23). Specific adverse effects, such as gastrointestinal disturbances and allergic reactions, were documented but were typically mild and manageable. The detailed findings are summarized in Table 1.

Table 1. Summary of the studies that assess effectiveness, cost considerations and safety issues associated with herbal medications in DM.

Reference	Type of Study, No. of Participants	Intervention	Outcome	Result	Cost Study	Safety Study
(21)	Randomized Controlled Trial (RCT); 52 participants (26 in each group)	Fenugreek (20g/day) and Cinnamon (4g/day)	Fasting Plasma Glucose (FPG), HbA1c, Insulin levels	Significant reduction in FPG and HbA1c in both groups; Insulin level fell significantly only in Cinnamon group	NA	NA
(22)	Prospective, single arm, uncontrolled, multicenter 30 participants	IND-2 (700 mg Fenugreek, thrice daily)	HbA1c levels, FBS, PPBS, insulin requirement	No significant reduction in FBS; HbA1c improvement noted	NA	Majority of adverse events were mild; 4 discontinuations due to AEs

Reference	Type of Study, No. of Participants	Intervention	Outcome	Result	Cost Study	Safety Study
(23)	Prospective observational study - 60 participants Pilot study - 41 participants Prospective cohort study - 94 participants Pilot study - 80 participants Randomized, double-blind, placebo-controlled clinical trial - 70 participants Participant-blinded, placebo-controlled clinical trial - 114 participants Double-blind, randomized controlled trial - 250 participants Single-blind, randomized controlled trial - 99 participants Non-randomized clinical trial - 114 participants Randomized clinical trial - 117 participants	*Nigella sativa* (Black Seed) oil - Doses ranging from 2 g/day to 1350 mg/day. Combination therapy with metformin. Placebo control in various trials. Oral N. sativa oil - 2.5 mL/day for chronic kidney disease patients. Powdered N. sativa - 2 g/day for 1 year. N. sativa oil - 1.5 mL and 3 mL daily for metabolic syndrome patients.	FBS and HbA1c levels.	Significant improvement in metabolic parameters such as HbA1c and FBG. Comparable effectiveness to metformin in some studies. Protective effects on cardiac function in diabetic patients. Statistically significant reductions in cholesterol levels. Significant reduction of insulin resistance and inflammatory markers.	NA	No major adverse effects reported in the studies. Safety observed in long-term administration (up to 1 year). Monitoring of metabolic parameters ensured participant safety throughout the trials.

Reference	Type of Study, No. of Participants	Intervention	Outcome	Result	Cost Study	Safety Study
(24)	Cross-sectional-739 participants	Herbal medicine use in combination with antidiabetic agents 1.Pandan leaf (*Pandanus amaryllifolius*) - 23.1% 2.Bitter gourd (*Momordica charantia*) - 19.2% 3.Country mallow (Abutilon indicum) - 10.8% 4.Yanang leaf (Tiliacora triandra) - 6.2% 5.Lingzhi mushroom (*Ganoderma lucidum*) - 6.2%	Glycemic control (HbA1c levels)	These plants were used in combination with prescribed antidiabetic drugs, and the study highlighted that bitter gourd, in particular, was associated with good glycemic control when used alongside 500 mg/day of metformin	NA	NA

Reference	Type of Study, No. of Participants	Intervention	Outcome	Result	Cost Study	Safety Study
(25)	Randomized controlled trial-62 participants	GanopolyTM (1800 mg three times daily for 12 weeks)	Fasting and postprandial plasma glucose levels, HbA1c	Reduced	NA	NA
	Randomized controlled trial-84 participants	G. lucidum (3 g/day) or G. lucidum plus Cordyceps sinensis capsules for 16 weeks	Hyperglycemia and cardiovascular risk factors	No significant effects	NA	NA
	Randomized, double-blind, placebo-controlled study-26 participants	Lingzhi (1.44 g extract/d for 12 weeks)	insulin and HOMA-IR (homeostasis model assessment–insulin resistance)	Reduced	NA	NA
(26)	Clinical Trial (e.g., 50-100)	*Nigella sativa* oil	Glycemic control (FBG, HbA1c, insulin levels)	Marked improvement in glycemic control and lipid profile after 8 weeks of treatment	NA	NA
	In Vivo Study-Not specified	Aqueous extract of *Nigella sativa*	Antidiabetic effect	Demonstrated antidiabetic effects in diabetic rats	NA	In vivo toxicity assessed

Reference	Type of Study, No. of Participants	Intervention	Outcome	Result	Cost Study	Safety Study
(5)	Clinical Study Not specified	*Allium sativum* (Garlic)	Blood sugar levels	Significant reduction in blood sugar levels observed	NA	Yes
	Clinical Study Not specified	*Momordica charantia* (Bitter Melon)	Blood sugar levels	Notable improvement in glycemic control	NA	Yes
	Clinical Study Not specified	*Hibiscus sabdariffa* L. (roselle)	Blood pressure and blood sugar	Positive effects on both blood pressure and blood sugar levels	NA	Yes
	Clinical Study Not specified	*Zingiber officinale* Rosc. (Ginger)	Blood sugar levels	Reduction in fasting blood glucose levels	NA	Yes

RCT = Randomized Controlled Trial; FPG = Fasting Plasma Glucose; HbA1c = Hemoglobin A1c; FBS = Fasting Blood Sugar; PPBS = Postprandial Blood Sugar; HOMA-IR = Homeostasis Model Assessment of Insulin Resistance; NA = Not Available.

Table 2 provides a comprehensive overview of the cultural, societal, professional, and regulatory factors that influence the use of herbal remedies in diabetes management. Key findings highlight the diverse motivations for using herbal remedies, including dissatisfaction with conventional treatments, cultural beliefs, and economic factors. Furthermore, the table explores the role of perceptions, communication, and obstacles in the adoption and utilization of herbal medicine. These factors collectively shape the complex landscape of herbal remedy usage in diabetes care.

Table 2. Cultural, Societal, Professional, and Regulatory Factors Affecting the Use of Herbal Remedies in Diabetes Management

Topic	Key Findings	References
Motivations	- Dissatisfaction with conventional treatments, preference for holistic approaches, cultural beliefs - Perceptions of herbal medicine as more natural or safer than pharmaceuticals - Economic factors, including cost of conventional treatments and perceived affordability of herbal alternatives - Access and availability of herbal practitioners and products - Enhancing quality of life and gaining a sense of control over health	(29,30)
Perceptions	- Cultural and ethnic factors influence acceptance and integration of herbal remedies - Regulation and professionalization of herbal medicine impact trust and usage - Evidence-based research and psychological factors (e.g., stress management, placebo effect) influence perceptions	(31,32)
Communication	- Many herbal medicine users do not disclose use to healthcare providers due to concerns about judgment, lack of support, and fears of negative reactions - Healthcare providers often do not routinely inquire about herbal medicine use, contributing to communication gaps - Patients often seek information on herbal remedies from sources other than healthcare providers	(33,34)
Obstacles	- Lack of standardized practices and consensus on efficacy and safety of herbal treatments - Inadequate provider education and training on herbal medicine, leading to skepticism and reluctance to recommend - Structural barriers in healthcare systems, including cost and time constraints, and lack of insurance coverage for herbal remedies - Cultural and societal factors influencing acceptance and use of herbal medicine - Regulatory challenges due to inconsistent standards, affecting product quality and efficacy	(35,36)

Using the Cochrane Collaboration's Risk of Bias tool, we assessed the methodological rigor of the studies. This evaluation focused on randomization, allocation concealment, blinding, and selective reporting to assess the reliability and validity of the findings. Table 3 summarizes these quality assessments, providing a clearer picture of the literature's methodological strength.

Table 3. Assessment of Bias Risks in Included Studies on Herbal Remedies for Diabetes Management

Reference	Study Type	Selection Bias	Performance Bias	Detection Bias	Attrition Bias	Reporting Bias	Overall Bias Risk
(21)	RCT	Low	Low	Low	Low	Low	Low
(22)	Uncontrolled	High	High	High	High	Medium	High
(23)	Observational	High	N/A	N/A	Unknown	Medium	Medium
(24)	Cross-sectional	High	N/A	N/A	N/A	High	High
(25)	RCT	Low	Low	Low	Low	Low	Low
(26)	Clinical Trial	Unknown	N/A	N/A	N/A	Unknown	Unknown
(5)	Clinical Study	Unknown	N/A	N/A	N/A	Unknown	Unknown

- **N/A**: Not Apply

4. DISCUSSION

4.1. Trends in Herbal Medicine Use for Diabetes

Our review of the literature reveals several key trends in the use of herbal medicine for diabetes management:

4.1.1. Motivations and Perceptions Regarding the Use of Herbal Medicine

A significant trend observed across studies is the multifaceted motivation behind herbal medicine use. Individuals often turn to herbal remedies due to dissatisfaction with conventional treatments, a preference for holistic approaches, or deeply rooted cultural beliefs. The perception of herbal medicines as "more natural" or safer than pharmaceutical options significantly influences its use (Table 2) (37,38).

4.1.1.1. Economic Factors

Multiple studies highlight the role of economic considerations in driving individuals towards herbal alternatives. The high cost of conventional diabetes treatments appears to be a significant factor influencing this trend, especially in low- and middle-income countries. This shift is especially evident when conventional treatments are not covered by insurance (37,39). These economic motivations are further reflected in the key findings of Table 2.

4.1.1.2. Prevalence and Cost of Herbal Medicine Use

Research shows that many individuals turn to herbal alternatives for diabetes management, particularly in low and middle-income countries where financial barriers limit access to conventional treatments (40,41). Americans spend over $34 billion annually out-of-pocket on Complementary and Alternative Medicine (CAM) therapies, reflecting a widespread perception that the perceived benefits of these treatments outweigh their costs (42). A comprehensive review by Alzahrani et al. revealed significant global variation in the use of CAM, with a prevalence rate of 51% and substantial heterogeneity. The most commonly used herbs include cinnamon, fenugreek, garlic, aloe vera, and black seed (20). In Thailand, 37.5% of patients used herbal medicine alongside their prescribed antidiabetic drugs, with tea being the most common form (24). In Africa, the use of traditional medicine ranges from 12.4% to 77.1%, with a median of 50% (43).

Despite the high prevalence, the financial and practical implications of herbal medicine use are complex. Initially, herbal remedies may appear cost-effective compared to conventional treatments (44,45). However, the true cost-effectiveness of these remedies involves more than just immediate out-of-pocket expenses. Herbal medicines often lack the comprehensive evidence and long-term effectiveness provided by conventional medicines. This can lead to potential costs down the line if the remedies prove less effective in managing chronic conditions like diabetes (46,47).

Literature reviews on the cost-effectiveness of herbal

medicine in diabetes management are notably scarce, reflecting a significant gap in our understanding of the economic implications of these interventions. While cost-effectiveness analyses (CEAs) have been conducted for herbal treatments in various other health conditions, comprehensive studies specifically addressing diabetes remain limited. This scarcity is particularly concerning given the global prevalence of diabetes and the potential role of herbal medicines in its management (45).

To contextualize this gap, it is instructive to consider examples from other therapeutic areas where CEAs of herbal medicines have been conducted. For instance, Lin et al. evaluated the long-term economic impact of combining traditional Chinese medicine (Gastroma Guteng Yin) with conventional western medicine for hypertension management. Their study revealed improved health outcomes but at significantly higher costs, underscoring the complexity of economic evaluations in herbal medicine (48). In the realm of mental health, St. John's wort has been shown to be as effective as conventional antidepressants but at a lower cost, demonstrating the potential for cost-effective herbal interventions (49). Similarly, in chronic pain management, acupuncture, often involving herbal remedies, has been found to be more cost-effective than conventional treatments for chronic low back pain (50). These examples highlight the feasibility and importance of conducting rigorous CEAs in herbal medicine.

However, the paucity of such studies in diabetes management represents a critical research gap (45). The multi-component nature of herbal products complicates toxicity assessments and efficacy evaluations, presenting unique challenges in conducting comprehensive CEAs (51). Furthermore, the heterogeneity of outcomes within herbal medicine research and the importance of considering local economic contexts add layers of complexity to these analyses (52). As diabetes imposes a significant economic burden on healthcare systems worldwide, there is an urgent need for well-designed, long-term studies that evaluate not only the clinical efficacy but also the cost-effectiveness of herbal interventions in diabetes management. Such research would provide valuable insights for policymakers, healthcare providers, and patients, potentially leading to more informed decision-making and resource allocation in diabetes care.

4.1.1.3. Access and Psychological Aspects

Multiple studies also highlight the importance of access and availability in the use of herbal medicine. In regions where herbal practitioners and products are more accessible, usage tends to be higher, with geographic and socioeconomic factors affecting access to both conventional and alternative therapies. Additionally, many patients use herbal medicine to enhance their quality of life and gain a sense of control over their health, finding empowerment in actively participating in their care (38,53). Psychological factors, including stress management and the placebo effect, contribute to the perceived benefits of herbal medicine, as these remedies can offer psychological comfort that positively affects health outcomes (54,55).

4.1.1.4. Cultural and Ethnic Influences

The literature consistently points to the strong influence of cultural and ethnic factors on herbal medicine use. Some communities have a long history of using herbal medicine, deeply rooted in cultural traditions (56,57). Additionally, some individuals combine herbal remedies with conventional treatments to enhance their overall health regimen, highlighting the role of herbal medicine in comprehensive patient care strategies (29).

4.2. Research Gaps and Methodological Challenges

Our review has identified several critical gaps in the current research landscape:

4.2.1. Safety Profiles of Herbal Medicines Used for Diabetes

There is a notable lack of rigorous scientific validation for the safety profiles of many herbal remedies used in diabetes management. This gap is particularly concerning given the potential for drug-herb interactions and liver

toxicity. The lack of rigorous scientific validation for the safety profiles of many herbal remedies is compounded by the bias risks present in the studies reviewed. Table 3 summarizes the assessment of bias risks, indicating that many studies, particularly observational ones, face significant limitations in their design and execution. The safety of herbal medicines used for diabetes management is a critical concern, particularly since many patients do not disclose their use of CAM to their healthcare providers (58). While the widespread use of herbal remedies among individuals with diabetes indicates a perceived safety and efficacy, specific details on their safety profiles are often not well-documented (59). This risk is further amplified in patients on polypharmacy, where the complexity of drug regimens increases the likelihood of harmful interactions, as demonstrated in national diabetes programs such as PROLANIS in Indonesia (60).

Numerous herbal remedies show promise in glycemic control, their safety profiles often lack rigorous scientific validation. For example, Teschke et al. highlighted the potential liver toxicity associated with certain Chinese herbal medicines used for diabetes (61). Fenugreek has been reported to cause hypoglycemia when used concurrently with insulin or sulfonylureas (62), while bitter melon may interact with P-glycoprotein and CYP3A4 substrate drugs, potentially altering their effectiveness (63). Ginseng has been associated with side effects such as headaches, insomnia, and hypertension (64).

Recent studies have found that, although some herbal medicines demonstrate hypoglycemic effects, they may also cause liver, kidney, and lung damage, highlighting the need for caution in their long-term use (65,66). The American Diabetes Association (ADA) emphasizes the importance of patient-provider communication regarding herbal supplement use, as failure to disclose such information can lead to dangerous drug-herb interactions (67). Quality control of herbal products is also a pressing issue, with studies revealing inconsistencies in active ingredient concentrations and occasional contamination with heavy metals or pharmaceutical agents (68). These concerns underscore the necessity for standardized manufacturing processes, comprehensive safety evaluations, and improved regulatory oversight in the herbal medicine industry.

Healthcare professionals often express reluctance to recommend herbal treatments due to concerns about interactions with conventional medications and insufficient knowledge about their safety profiles (69). While herbal remedies can be gentler than synthetic drugs, they are also prone to issues such as plant misidentification, incorrect preparation, and improper administration by inadequately trained practitioners. In contrast, synthetic drugs, though newer to Western medicine, are generally trusted more due to their standardized production and well-documented effects. Therefore, ongoing research and careful monitoring are essential to address safety concerns and improve patient outcomes with herbal medicine (70).

4.2.2. Long-term efficacy studies

There is a scarcity of long-term, large-scale clinical trials to conclusively establish herbal medicine efficacy in diabetes management (45,71). This poses significant challenges to establishing the efficacy and safety of these treatments. Current research is limited by small sample sizes, short durations, and varying methodologies, making it difficult to draw definitive conclusions about the effectiveness of herbal remedies (51). This lack of robust evidence is a major barrier to the integration of herbal medicine into mainstream diabetes care, as healthcare providers and policymakers require high-quality data to make informed decisions. The absence of comprehensive clinical trials limits the understanding of how herbal medicines interact with conventional diabetes treatments, particularly given the complex nature of diabetes management. Large-scale studies could provide valuable insights into the synergistic effects of combining herbal and conventional therapies, potentially leading to more effective and personalized treatment strategies (72).

Addressing these research gaps is crucial for advancing the field and ensuring patients have access to safe and effective treatment options.

4.2.3. Standardization and Quality Control

The literature highlights significant challenges in the standardization and quality control of herbal products, which complicates the assessment of their safety and efficacy. Herbal medicines frequently face issues with standardization and quality. The variability in effectiveness and safety due to lack of standardization can impact their overall cost-effectiveness (73). Many countries lack stringent regulations for herbal drugs, leading to issues like adulteration and spurious products, which compromise safety and efficacy (74). In contrast, conventional medicines undergo rigorous testing and standardization processes to ensure consistency and reliability (75).

4.2.4. Communication with Healthcare Providers Regarding Herbal Medicine Use

A recurring theme in the literature is the communication gap between patients and healthcare providers regarding herbal medicine use. Effective communication between herbal medicine users and healthcare providers is crucial for ensuring safe and coordinated care. The review reveals that many individuals using herbal remedies do not disclose this information to their healthcare providers, primarily due to concerns about judgment, perceived lack of support, and fears of negative reactions (30,32,76). This reluctance complicates diabetes management because nondisclosure can lead to risks such as adverse drug interactions and hampers the integration of herbal remedies into conventional treatment plans (33). Notably, this pattern of nondisclosure and self-medication is not limited to herbal remedies; it extends to other medications, as demonstrated by a recent study in Lebanon showing that caregivers frequently administer antibiotic suspensions to children without consulting healthcare professionals (77). This suggests a deeply rooted culture of self-medication that underscores the need for targeted education and improved patient-provider dialogue.

The review also highlights that a significant number of healthcare providers do not routinely inquire about herbal medicine use, which contributes to this communication gap (45). Furthermore, only a small percentage of herbal users seek information from conventional practitioners, indicating that healthcare providers are not the primary source of information on herbal remedies (78).

Addressing these communication gaps is essential for improving patient safety and the effectiveness of treatment plans. The World Health Organization emphasizes the need for enhanced dialogue and collaboration between patients and healthcare providers to ensure the informed and safe use of herbal medicine (36). Initiatives that empower pharmacy students as health educators—such as training them to help patients interpret food labels and make informed decisions—demonstrate a successful model for building health literacy in Lebanon (79). This educational approach can be expanded to include counseling on herbal remedies, equipping future pharmacists to bridge the information gap and support safe, evidence-based self-management.

4.3. Integrating Findings: A Narrative Synthesis

Synthesizing the available literature reveals several key themes:

4.3.1. Efficacy of Specific Herbal Remedies

The effectiveness and safety of herbal medicines for diabetes management vary widely. Salleh et al. evaluated the impact of specific herbal remedies including turmeric, garlic, bitter melon, and roselle (*Hibiscus sabdariffa*) on blood glucose levels. Their findings revealed that a combination of turmeric and garlic significantly reduced fasting blood glucose levels and HbA1C, without adverse effects on blood pressure, liver, or kidney function. In contrast, bitter melon did not show significant effects on blood glucose or other health markers, which might be attributed to variations in bioactive compounds based on regional differences (80).

Zhang et al. reviewed Chinese Herbal Medicine (CHM)

for diabetic kidney disease, focusing on herbs such as Astragali Radix, Rehmanniae Radix, and Rhei Radix et Rhizoma. CHM was found to be beneficial as an adjunct therapy with renin-angiotensin system inhibitors, leading to reduced albuminuria and improved estimated glomerular filtration rate. However, the studies had limitations, including high heterogeneity and small sample sizes, which warrant cautious interpretation of the results (81).

Table 1 summarizes various studies on the use of medicinal plants in diabetes management. Ahmad et al. found that both fenugreek and cinnamon significantly reduced fasting plasma glucose and HbA1c, with cinnamon also lowering insulin levels (21). Kandhare et al. reported improvements in HbA1c with fenugreek but noted mild adverse events and no significant reduction in fasting blood sugar (22). Maideen showed that *Nigella sativa* oil improved metabolic parameters and was comparable to metformin, with no major adverse effects (23). Prasopthum et al. highlighted that bitter gourd, when used with metformin, improved glycemic control (24). Chan et al. observed mixed results with *Ganoderma lucidum*, including reduced glucose levels in some trials (25). Adam et al. demonstrated significant glycemic and lipid profile improvements with *Nigella sativa* (26), while Yedjou et al. noted positive effects on blood sugar levels and safety for garlic, bitter melon, roselle, and ginger (5). Overall, the studies suggest that these herbs can positively impact diabetes management, although safety and cost evaluations vary. However, most of these studies focus exclusively on biochemical and clinical markers, with little to no assessment of patient-reported outcomes such as health-related quality of life (HRQoL). A recent study in Quetta, Pakistan, demonstrated the value of using the EQ-5D-3L instrument to evaluate HRQoL in patients with Type 2 Diabetes, revealing significant impacts of the disease on daily functioning and well-being (82). Future research on herbal remedies should incorporate such validated tools to provide a more comprehensive understanding of their true impact on patients' lives.

4.3.2. Regulatory Challenges

The review reveals a global challenge in regulating herbal medicines, with significant variations in regulatory frameworks across different countries. This inconsistency impacts the quality, safety, and efficacy of herbal products available to diabetes patients. In countries where herbal medicine is regulated and practitioners are licensed, there is often higher trust and usage of these remedies. In India, the AYUSH system regulates nearly 8000 herbal medications, fostering trust in their safety and effectiveness (83). Regulatory challenges pose significant barriers as well. Herbal medicine manufacturers and practitioners often face difficulties due to inconsistent regulatory standards, resulting in variability in product quality and efficacy. This lack of uniform regulation complicates efforts to integrate herbal medicine into established healthcare frameworks (35,84,85).

Case studies provide valuable insights into these challenges. For example, a study in India highlighted how regulatory inconsistencies led to the prevalence of low-quality herbal products, affecting patient safety and treatment outcomes (35). Conversely, in Germany, well-regulated herbal medicine practices within the healthcare system have demonstrated successful integration, showcasing how standardized approaches can enhance both safety and efficacy (86).

Comparative analysis reveals different approaches taken by other countries. In China, herbal medicine is widely integrated into the healthcare system, supported by robust regulatory frameworks and research initiatives (87). Similarly, in South Korea, the integration of traditional medicine into conventional healthcare has been facilitated by government support and standardized practices, providing a model for other regions (88).

4.3.3. Socioeconomic and Cultural Determinants

The literature consistently points to the significant role of socioeconomic factors and cultural beliefs in shaping patients' choices regarding herbal medicine use. Cultural beliefs and societal attitudes can strongly impact

individuals' preferences for herbal remedies over conventional treatments. For example, in many communities, herbal medicine is deeply ingrained in tradition and is considered a trusted component of healthcare (36,89). Vinca rosea, also known as *Catharanthus roseus*, is a traditional medicine in Jordan that is used to manage diabetes. Research has shown that leaf extracts from this plant have antidiabetic properties (90). Local herbalists prescribe remedies based on symptoms rather than a comprehensive understanding of the underlying conditions. The Northern Badia region, known for its diverse medicinal plants, stands out for its unique herbal practices (41). This highlights the need for culturally sensitive and economically considerate approaches in diabetes care.

4.3.4. Obstacles to Integrating Herbal Medicine into the Healthcare System

Integrating herbal medicine into the healthcare system encounters several notable obstacles. A key challenge is the lack of standardized practices and consensus on the efficacy and safety of various herbal treatments (73). Many healthcare providers are inadequately trained to discuss herbal medicine with their patients, leading to missed opportunities for addressing potential drug-herb interactions and ensuring patient safety. This training gap, along with limited clinical data and research specifically on herbal medicine, contributes to provider skepticism and reluctance to recommend these therapies (34,91). The absence of insurance coverage for herbal remedies and therapies further complicates integration efforts (92).

Future directions for overcoming obstacles in herbal medicine promotion include enhancing provider education, improving regulatory standards, and increasing clinical research to build a stronger evidence base, as suggested in the literature (93). In summary, while the integration of herbal medicine into the healthcare system faces significant challenges—such as skepticism, inadequate provider education, regulatory issues, and cultural factors—there are also promising avenues for improvement (94). By learning from successful international models and addressing these obstacles proactively, the potential benefits of herbal medicine can be better realized and incorporated into mainstream healthcare (36).

5. CONCLUSION

The use of medicinal plants like cinnamon, aloe vera, bitter gourd, turmeric, garlic, and roselle flower in diabetes treatments can enhance glucose control and overall health. However, it is crucial to educate healthcare providers and patients about potential interactions between herbal remedies and prescribed anti-diabetic medications. These efforts require updating diabetes management practices based on scientific evidence, involving patients and caregivers, and integrating herbal medicine into national health systems. Further research on herbal medicine is important for establishing safety profiles and promoting evidence-based guidelines. Encouraging lifestyle modifications and a whole food plant-based diet can also help manage diabetes and prevent complications.

6. Conflicts of Interests

The authors declare that there are no conflicts of interest.

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مراجعة أدبية للعلاجات العشبية المستخدمة في داء السكري: الفعالية، السلامة، والاعتبارات التنظيمية

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ملخص

المشكلة: إن الانتشار المتزايد لداء السكري من النوع الثاني يشكل عبئاً صحياً واقتصادياً كبيراً على مستوى العالم، مما أدى إلى تنامي الاهتمام بالطب العشبي كخيار بديل أو مكمل منخفض التكلفة للعلاجات التقليدية.

المنهج التجريبي : شملت هذه المراجعة الأدبية دراسات مُحكّمة نُشرت بين عامي 2018 و2024. تم إجراء بحث شامل في قواعد بيانات مثل PubMed و Scopusو Google Scholarباستخدام مصطلحات مرتبطة بالسكري، والطب العشبي، والجدوى الاقتصادية. وقد شملت الدراسات المضمّنة بلداناً متعددة منها الهند، الصين، الولايات المتحدة، المملكة المتحدة، السعودية، وعدد من الدول الإفريقية. تم تضمين الدراسات التي استهدفت البالغين المصابين بالسكري وقيّمت فعالية العلاجات العشبية أو تكلفتها أو تجارب المستخدمين. واستُبعدت الدراسات التي شملت أطفالاً، أو لم تكن مُحكّمة، أو لم تكن مرتبطة بشكل مباشر بالطب العشبي. وقد أجرى باحثان عملية استخلاص البيانات بشكل مستقل باستخدام نموذج موحّد لجمع معلومات حول خصائص الدراسة، وحجم العينة، والعلاجات العشبية، ونتائج التحكم في نسبة السكر في الدم، والآثار الجانبية.

النتائج الرئيسية :بلغ عدد الدراسات المشمولة 45 دراسة، تضمنت تجارب سريرية عشوائية ودراسات رصدية. أشارت النتائج إلى أن بعض العلاجات العشبية يمكن أن تحسن التحكم في نسبة السكر في الدم بشكل ملحوظ، مع متوسط انخفاض في HbA1c يتراوح بين 0.5% و1.5%. كما أظهرت التأثيرات الاقتصادية لهذه العلاجات مزايا محتملة من حيث التكلفة مقارنة بالعلاجات التقليدية، على الرغم من أن ملفات السلامة أظهرت بعض الآثار الجانبية المصاحبة.

الاستنتاجات :تُبرز هذه المراجعة الفوائد المحتملة للعلاجات العشبية في إدارة داء السكري من النوع الثاني، لكنها تؤكد في الوقت نفسه على الحاجة إلى مزيد من البحث. وينبغي أن تركز الدراسات المستقبلية على الجرعات المثلى، والتفاعلات مع الأدوية التقليدية، والجدوى الاقتصادية، وسلامة وفعالية الاستخدام على المدى الطويل، وخاصة من خلال التجارب السريرية العشوائية ذات الأحجام الكبيرة وفترات المتابعة الممتدة.

الكلمات الدالة: الطب العشبي؛ داء السكري من النوع الثاني؛ الجدوى الاقتصادية؛ التحكم في نسبة السكر في الدم؛ ملفات المستخدمين؛ التأثير الاقتصادي.

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The Relationship between Diabetic Patients' Health Literacy and HBA1c Level in Jordan

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ABSTRACT

Objective: This study aimed to explore how diabetic health literacy influences glycated hemoglobin HbA1c levels—a crucial marker of long-term blood sugar control—in Jordanian patients with type 2 diabetes.

Methods: Over a four-month period at a major public hospital in Amman, we enrolled 400 patients with type 2 diabetes mellitus in this cross-sectional study. The study used the Jordanian Diabetic Health Literacy Questionnaire (JDHLQ). This validated tool assesses health literacy among Arabic-speaking individuals.

Results: The findings revealed a significant link between higher health literacy scores and lower HbA1c levels. Specifically, each additional point on the JDHLQ was associated with a 0.040 decrease in HbA1c (95% CI [-0.078, -0.003], p=0.035). Patients taking more medications and those without insurance also had significantly higher HbA1c levels.

Conclusion: These results highlight the vital role of health literacy in managing diabetes effectively and support the implementation of targeted educational programs to improve patient outcomes in Jordan. The study emphasizes the need for policy improvements in diabetes care.

Practice Implications: Understanding the key factors that influence disease control in type 2 diabetes patients—including the impact of health literacy—is essential for developing targeted interventions, enhancing patient outcomes, and reducing the strain of diabetes on the healthcare system.

Keywords: Health literacy; Diabetes Miletus; Jordan; HBA1c, glycemic control.

1. INTRODUCTION

Diabetes mellitus (DM) is primarily characterized by abnormally high blood glucose levels [1], which significantly increase the risk of microvascular complications such as neuropathy, nephropathy, and retinopathy. In addition, DM is linked to a higher likelihood of macrovascular complications, including peripheral vascular disease, stroke, and ischemic heart disease. These health issues contribute to substantial morbidity, a decreased life expectancy, and a diminished quality of life for those affected [2–4].

Based on four population-based surveys conducted in 1994, 2004, 2009, and 2017, the prevalence rate of DM in

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Jordan was 12.9%, 17.4%, 20.8%, and 22.4%, respectively. Thus, the prevalence is increasing at an alarming rate [5]. This increase represents a significant burden on healthcare services in Jordan. Globally, DM constitutes a serious public health problem that has significant economic consequences in addition to adverse health impacts [6]. In 2010, the global prevalence of DM among adults was 285 million patients. It is estimated to rise to 439 million by 2030. The projected increase between these two years is 20% in developed countries and 69% in developing countries [7].

Health literacy (HL) is extremely important and plays an essential role in the development of health, economic, and social sectors [8]. Diabetic HL is the patient's ability to search for, perceive, analyze, and apply DM-related information in both daily lives and healthcare settings [9]. Findings from a cross-sectional study conducted in May 2019 in Ethiopia revealed that adequate diabetic HL is strongly correlated with better glycemic control. Moreover, after adjusting for all variables including high diabetic HL, younger age, and adequate adherence, these factors were found to be associated with achieving optimal glycemic control[10]. In addition, results from a US cross-sectional study conducted in 2000 indicated that poor HL was independently associated with greater rates of retinopathy and poor glycemic control [11].

Addressing the factors that influence glycemic control is central to preventing complications associated with diabetes [12]. A cross-sectional study conducted across three hospitals in northern Jordan discovered that a significant number of participants had inadequate self-care behaviors and poor glycemic control [13]. Another Jordanian cross-sectional study found that the prevalence of poor glycemic control among participants was significantly high [14,15].

Healthcare professionals often use the hemoglobin A1c (HbA1c) test to assess glycemic control in patients with type 2 diabetes mellitus (T2DM), as HbA1c levels are critical indicators of potential diabetic complications [16,17]. This test reflects an individual's average blood glucose levels over the preceding two to three months [18].

Previous studies [19,20] have suggested a strong link between diabetic patients' health literacy, their level of knowledge, and the achievement of better disease control. However, to date, no research in Jordan has examined the relationship between health literacy and disease management, including HbA1c levels, among diabetic patients. It is essential to understand the key factors that influence disease control in the Jordanian population in order to develop targeted interventions, improving patient outcomes, and reducing the burden of diabetes on the healthcare system. Therefore, this study aimed to investigate the relationship between health literacy and disease control, including HbA1c levels, in T2DM patients in Jordan

2. METHODS

A total of 400 patients with diabetes mellitus who were attending the endocrinology outpatient clinic in a major public tertiary hospital in eastern Amman, Jordan, were recruited. Data collection took place between August and November 2023. Participants were required to be adults aged 18 or older, have a diagnosis of type 2 diabetes mellitus for at least one year, and provide written informed consent to join the study. The research team reviewed the medical records of patients scheduled for follow-up appointments the next day to identify those who met the inclusion criteria. Eligible patients were approached on the day of their appointment by trained researchers (Z.A & S.A). The researchers explained the study's objectives, assured them of the confidentiality of their data, and informed them of their right to withdraw from the study at any time. The self-administered questionnaire took about ten minutes, and participants were informed of this beforehand. All participants gave informed consent and signed a consent form. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Ethical approval was obtained from the Al-Zaytoonah University of Jordan (Ref#18/09/2022–2023).

2.1. Data collection and study instruments

This study used the Jordanian Diabetic Health Literacy Questionnaire (JDHLQ), a validated instrument for assessing DM patients' health literacy in Arabic-speaking populations [21]. Additionally, sociodemographic information was collected, including gender, age, marital status, educational level, and monthly income. Other information was obtained from patients' medical files, including the medication used and HbA1c readings on the day of the visit.

The JDHLQ is composed of two main parts. The first part targets the informative aspect of health literacy, assessing how well patients with T2DM can understand, evaluate, and apply information related to their condition. The informative domain contains five items. The second section assesses the communicative domain of HL and is composed of three items evaluating DM patients' ability to explain their health condition to healthcare professionals, explain the importance of a special diabetic diet, and their ability to effectively ask healthcare professionals questions (Appendix A). according to the achieved scores of JDHLQ, participants were classified into "High" or "Low" health literacy based on 80% Bloom's cut-off point.

Adherence to medications was measured using the Medication Adherence Report Scale questionnaire (MARS-5); a self-report tool with validated Arabic version [22]. MARS-5 is a frequently utilized tool to assess adherence to medications in chronic diseases. It consists of five items: "I forget to take them", "I change the dose", "I stop taking them for a while", "I decide to skip a dose", and "I take medications less than instructed". Reponses are designated as "always", "often", "sometimes", "rarely" and "never". The total score accordingly ranges from 5 to 25, with a higher score indicating better adherence to medication. (Appendix A).

Answers to the items in both sections are based on a four-point Likert scale with the highest possible score being 32. The higher the total score, the better the diabetic HL.

2.2. Sample size calculation.

The 50 + 8P equation [23] was employed to calculate the minimum required sample size to generate a regression model with an adequate level of statistical power. P in the equation indicates the number of predictors. The current study investigated the association of 12 variables with the diabetic control score. Therefore, the minimum required sample size was 146 patients.

2.3. Statistical analysis

All statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) software version 26. Continuous variables were summarized as medians with interquartile ranges (25th to 75th percentiles), and categorical variables were presented as frequencies and percentages. To assess the relationship between the diabetic control score (the outcome variable) and various factors—including gender, age, marital status, education level, monthly income, insurance status, medications (Metformin, Insulin, Sulfonylureas, and DPP-4 inhibitors), and the JDHLQ score—we conducted a quantile regression analysis. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

We enrolled a total of 400 patients out of 442 (response rate was 90.5%) with diabetes mellitus in this study, with women comprising 68.8% of the participants. Detailed sociodemographic profiles are presented in Table 1. The median age of the participants was 58 years, ranging from 50 to 64 years, and the vast majority were married (89.2%). Most participants (79.0%) had health insurance, and a significant portion had completed only elementary education (42.5%). A large majority (81.2%) reported a monthly household income of less than 500 Jordanian Dinars (about 705 US dollars). Metformin was the most commonly used antidiabetic drug (86.7%), followed by insulin (37.7%). Thiazolidinediones (TZDs) were the least commonly used, with only 1.8% of participants taking them. The median score of the JDHLQ was 22 out of 32 (68.7%), with scores ranging from 18 to 25. The median of the MARS-5 adherence score was 21 (19-25).

Table1. Sociodemographic characteristics of diabetic patients (n=400).

		Median (percentile 25-75)	Count (%)
Age		58(50-64)	
HbA1c		8.00 (6.80-10.00)	
Gender	Female		275 (68.8%)
	Male		125 (31.3%)
Education	Elementary		169 (42.5%)
	High school		142 (35.7%)
	College/university degree		87 (21.9%)
Marital status	Single		43 (10.8%)
	Married		355 (89.2%)
Monthly income	less than 500 JD		323 (81.2%)
	500 JD or more		75 (18.8%)
Do you have health Insurance?	No		84 (21.0%)
	Yes		316 (79.0%)
Medicationsa	Insulin		150 (37.7%)
	Metformin		345 (86.7%)
	DPP-4 inhibitorsb		59 (14.8%)
	GLP-1-and dual GLP-1 GIP receptor agonistsc		15 (3.8%)
	SGLT2-Inhibitorsd		12 (3%)
	Sulfonylureas		38 (9.5%)
	Thiazolidinediones (TZDse)		7 (1.8%)
JDHLQ score		22(18-25)	
JDHLQ category	High	89 (22.2)	
	Low	311 (77.8)	
MARS-5 score		21 (19-25)	
HbA1c		8 (6.7-9.93)	

a; recruited patients were on one or more hypoglycemic therapy

b: Dipeptidyl Peptidase IV inhibitors

c: Glucagon-like peptide-1 and Gastric inhibitory polypeptide receptor agonists

d: Sodium-Glucose Transport Protein 2 (SGLT2) Inhibitors

JD: Jordanian dinars, equivalent to $1.41

A quantile regression was conducted to determine variables associated with the HbA1c levels. Results are displayed in Table 2. Higher JDHLQ scores were significantly associated with lower HbA1c (-0.050, 95% CI [-0.089, -0.011], p=0.011). In addition, patients taking a larger number of medications had significantly higher HbA1c levels (1.131, 95% CI [0.445, 1.807], p<0.001). Moreover, patients who did not have insurance were found to have significantly higher HbA1c levels (0.615, 95% CI [0.118,1.112], p=0.015). Finally, patients who did not take insulin had significantly lower HbA1c levels than those who took insulin (-1.433, 95%CI [-2.137,-0.692], p<0.001).

Table 2. Quantile regression analysis of variables influencing the diabetic control score.

Parameter		Coefficient	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
(Intercept)		5.996	<0.001	2.685	9.308
Age		-0.003	0.706	-0.020	0.013
JDHLQ		-0.050	0.011	-0.089	-0.011
Number of medications		1.131	0.001	0.455	1.807
Adherence score		0.029	0.269	-0.022	0.080
Gender	Female	0.038	0.867	-0.408	0.483
	Male	REF	.	.	.
Educational level	Elementary school	0.311	0.266	-0.238	0.861
	High school	0.129	0.644	-0.421	0.680
	Postgraduate education	REF	.	.	.
Marital status	Single	-0.050	0.884	-0.727	0.626
	Married	REF	.	.	.
Income	500JOD or less	0.591	0.031	0.056	1.126
	More than 500jOD	REF	.	.	.
Health insurance status	No	0.615	0.015	0.118	1.112
	Yes	REF	.	.	.
Insulin	High school	-1.433	<0.001	-2.173	-0.692
	Postgraduate education		.	.	.
Metformin	No	0.858	0.060	-0.036	1.751
	Yes	REF	.	.	.
DPP-4 inhibitors[a]	No	0.633	0.154	-0.238	1.505
	Yes	REF	.	.	.
Sulfonylureas	No	0.478	0.308	-0.443	1.398
	Yes	REF	.	.	.

a: Dipeptidyl Peptidase IV inhibitors

REF=Reference group

4. DISCUSSION

The prevalence of DM is increasing worldwide due to factors such as poor diet, sedentary lifestyle, aging populations, and limited access to medical care and health-related information [24]. The increased incidence of DM is particularly pronounced in Jordan [25]. DM can contribute to various complications in addition to lower life expectancy. Therefore, identifying and managing factors that contribute to poor glycemic control in Jordanian patients is of central importance. This study aimed to investigate the relationship between Jordanian diabetic patients' health literacy (HL) and HBA1c levels, in addition to exploring other factors significantly associated with HBA1c level.

Our results showed that higher levels of diabetic health literacy are linked to lower HbA1c values, evidencing better glycemic control. Health literacy in diabetic patients influences their medication adherence, self-care behaviors, and health-seeking actions—all of which are central factors affecting disease progression and glycemic management[26]. These findings are consistent with previous studies, including cross-sectional research conducted in countries like the USA [11], Saudi Arabia [27], Ethiopia[26], Denmark [28], and South Korea [29]. Moreover, earlier literature has shown that interventions focused on improving health literacy have led to significant improvements in HbA1c levels [30].

Our study found that patients had inadequate diabetes health literacy, with a median score corresponding to 68.7%. Health literacy significantly impacts patients' behavior, including medication adherence and self-care practices. Therefore, evaluating and enhancing patients' health literacy through various strategies can substantially improve outcomes for individuals with DM. Developing and implementing culturally tailored programs to elevate diabetic-related health literacy is essential [21].

[18] In the present study, insulin usage was associated with higher HBA1c levels. This can be explained by the fact that, according to the guidelines, insulin is prescribed to T2DM patients who are unable to achieve recommended glycemic control with first-line therapy. Therefore, insulin is generally reserved for more advanced and refractory cases of T2DM [31], as due to the progressive nature of the disease, multiple antidiabetic medications may be required to achieve recommended glycemic control [32].

Poor medication adherence is a crucial factor that leads to poor glycemic control and higher HBA1c levels [33]. Lower adherence to insulin therapy has been well-documented in previous studies [34]. Barriers that hinder DM patients' insulin adherence include being away from home, feeling embarrassed to inject insulin in public, fear of hypoglycemia, difficulty remembering to get refills from the clinician or to pick them up from the pharmacy, depression, health beliefs about medications, fear of pain, the burden of multiple daily dosages, cost, side effects, and the inherent complexity of insulin regimens [34,35]. However, implementing shared decision-making in developing an individualized treatment plan has been shown to enhance patient adherence [36]. Moreover, communicating these barriers to healthcare practitioners is a significant way to address them promptly [35].

In addition to medication adherence, healthcare professionals need to evaluate patients' adherence to self-care practices such as checking one’s feet, following a healthy diet, engaging in physical activity, and monitoring blood glucose levels[37]. The observed relationship between the use of a higher number of oral glucose-lowering medications and insulin, and increased HbA1c levels, could be explained by a lack of regular assessments of adherence to self-care practices by healthcare providers before increasing the medication dosage.

The current study found that patients without health insurance had higher HBA1c levels compared to insured patients. This is in line with findings of a Canadian study [38], and a study conducted in the USA [39]. Thus, healthcare costs are a prominent barrier to medication adherence among DM patients which may impact treatment outcomes.

To summarize, the present study found that health literacy, insulin use, number of medications used, and health insurance status were factors significantly associated with patients' HbA1c levels, which cumulatively affected disease outcomes.

The present study has generated novel findings in the field of diabetic-related health literacy in Jordan. These findings are of great importance considering the lack of similar research in Jordan and the broader region. The findings shed light on the gaps in the field and address critical factors involved in diabetes management. Furthermore, the study provides recommendations tailored to the needs of the targeted population.

Nevertheless, some limitations need to be acknowledged. Apart from the information obtained from patient records, the present study relied on self-reported data, exposing the findings to recall and social desirability biases. Furthermore, it is possible that patients who found the study objectives interesting were more likely to participate, which could have led to selection bias, which may lead to skewness in the sample demographics, for example most of the current participants were females. Furthermore, the present study did not assess other factors that may impact glycemic levels, such as depression. Nevertheless, the main focus of this research was to assess the association between health literacy and glycemic levels. Additionally, although the data were collected from a single hospital, it was one of the country's largest medical facilities and served a substantial number of patients from various geographic regions. This diversity allowed for a broad sample, potentially enhancing the generalizability of our findings.

5. CONCLUSION

This study closely examined how diabetic health literacy influences glycemic control, specifically HbA1c levels, in Jordanian patients with T2DM. The results show that health literacy is a key factor in effectively managing T2DM. Patients who understood their condition better tended to have lower HbA1c levels, indicating that improving health literacy could lead to better blood sugar control.

These findings are particularly important because there is limited research on this topic in Jordan and the surrounding region. They offer practical insights that healthcare providers and policymakers can use to develop strategies aimed at enhancing health literacy and improving diabetes management for patients in Jordan.

The limitations noted, such as the reliance on self-reported data and potential selection bias, suggest areas for further research. Future studies could incorporate more objective data collection methods and a broader participant base to confirm and expand upon these findings. Overall, enhancing diabetic health literacy remains a key strategy in the battle against diabetes in Jordan, with the potential to significantly reduce the burden of the disease on both patients and the healthcare system.

5.1. Practice implications

Investigating the prominent factors that have a significant role in disease control among T2DM patients in Jordan is essential for constructing well-structured targeted interventions in order to improve patient outcomes and reduce the burden of diabetes on the healthcare system.

Since health literacy greatly affects patients' behavior, assessing and improving patients' health literacy can significantly enhance diabetic patient outcomes. Therefore, applying culturally tailored programs to elevate diabetic-related health literacy levels is of great importance.

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CRediT authorship contribution statement

Abdel Qader Al Bawab: Writing – review & editing, Writing – original draft, Methodology, Conceptualization, Supervision. Walid Al-Qerem: Writing – review & editing, Writing – original draft, Methodology,

Investigation, Formal analysis, Data curation, Conceptualization, Supervision. Anan Jarab: Writing – review & editing, Investigation, Formal analysis, Conceptualization. Judith Eberhardt: Writing – review & editing, Investigation, Formal analysis. Fawaz Alasmari: Writing – review & editing, Formal analysis. Alaa Hammad: Writing – review & editing, Methodology, Conceptualization. Safa M. Alkaee: Writing – review & editing, Methodology, Conceptualization. Zein H. Alsabaa: Writing – review & editing, Methodology, Formal analysis, Conceptualization.

Institutional Review Board Statement:

The study followed the Declaration of Helsinki ethical guidelines. Ethical approval was secured from Al-Zaytoonah University of Jordan (Ref#18/09/2022–2023).

Informed Consent Statement:

"Written informed consent has been obtained from the patient(s) to publish this paper".

Data Availability Statement:

The dataset supporting the conclusions of this article is available in the Zenodo repository, https://doi.org/10.5281/zenodo.11081022

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العلاقة بين محو الأمية الصحية لمرضى السكري ومستوى HBA1c في الأردن

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ملخص

المقدمة: تهدف هذه الدراسة إلى استكشاف كيفية تأثير محو الأمية الصحية لمرضى السكري على مستويات الهيموجلوبين السكري - HbA1cوهي علامة حاسمة للتحكم في نسبة السكر في الدم على المدى الطويل -لدى المرضى الأردنيين المصابين بداء السكري من النوع 2.

المنهجية: على مدى أربعة أشهر في مستشفى عام كبير في عمان، قمنا بتسجيل 400مريض مصاب بداء السكري من النوع 2في هذه الدراسة المقطعية .استخدمت الدراسة استبيان محو الأمية الصحية الأردني لمرضى السكري .(JDHLQ) تقوم هذه الأداة التي تم التحقق من صحتها بتقييم محو الأمية الصحية بين الأفراد الناطقين باللغة العربية .

النتائج :كشفت النتائج عن وجود صلة كبيرة بين درجات محو الأمية الصحية المرتفعة وانخفاض مستويات HbA1c. على وجه التحديد، ارتبطت كل نقطة إضافية في JDHLQ بانخفاض 0.040 في 95) HbA1c٪ -0.078 CI [-0.003، - 0.035 = p). المرضى الذين يتناولون المزيد من الأدوية وأولئك الذين ليس لديهم تأمين لديهم أيضا مستويات أعلى بكثير من HbA1c.

الخلاصة :تسلط هذه النتائج الضوء على الدور الحيوي لمحو الأمية الصحية في إدارة مرض السكري بشكل فعال ودعم تنفيذ البرامج التعليمية الهادفة لتحسين نتائج المرضى في الأردن .وتؤكد الدراسة على الحاجة إلى تحسين السياسات في رعاية مرضى السكري .الآثار المترتبة على الممارسة :يعد فهم العوامل الرئيسية التي تؤثر على السيطرة على المرض لدى مرضى السكري من النوع - 2بما في ذلك تأثير محو الأمية الصحية -أمرا ضروريا لتطوير التدخلات المستهدفة، وتعزيز نتائج المرضى، وتقليل ضغط مرض السكري على نظام الرعاية الصحية.

الكلمات الدالة: محو الأمية الصحية، داء السكري من النوع الثانى، الأردن، التحكم فى نسبة السكر فى الدم.

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Effect of Lignin and other Biopolymers on Hyperlipidemia and Gut Microbiota

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ABSTRACT

So far, dietary fibers such as lignin, cellulose, pectin, guar gum, and psyllium have been well-studied for their preventive and therapeutical potential using animal and human models, especially for their beneficial effects on chronic metabolic conditions like dyslipidemia and related disorders. Dyslipidemia is a dangerous metabolic disorder related to hypercholesterolemia, coronary artery disease, and coronary heart disease. Earlier research has demonstrated that these dietary fibers can lower high serum lipid levels through different mechanisms. One of the most important mechanisms is the modification of gut microbiota. Increasing the abundance of lactic acid bacteria (LAB), which can metabolize different dietary fibers like lignin, may potentially reduce the cholesterol level. This review aims to provide useful insights and comprehensive discussions about current knowledge related to the properties, and the effects of dietary fibers mainly lignin in controlling hyperlipidemia and their effects on gut microbiota. Google Scholar, Research Gate, and Scopus are the search engines exploited to collect data by using lignin, biopolymers, gut microbiota, and hyperlipidemia as search terms.

Keywords: Lignin, biopolymers, gut microbiota, hyperlipidemia.

1. INTRODUCTION

Dietary fibers are the general term for lignin and polysaccharides (e.g. cellulose, mucus, and gums) [1]. These fibers can alter gastrointestinal function from the mouth to the anus [2] because a large amount of them are non-digested and non-absorbed plant carbohydrates by endogenous enzymes in the human small intestine [2]. But they undergo bacterial fermentation in the large intestine which affects the amount and species composition of the microbiota [1-3]. This fermentation process improves the health of the gut and different organs via the production of bioactive metabolites such as short-chain fatty acids that lead to changes in the immune system responses, reduction of intracolonic pH, and modulations of blood lipid levels [2].

Hyperlipidemia is described by extreme levels of lipids (low-density lipoprotein (LDL), triglycerides (TGs), and total cholesterol (TC)) in the blood. High plasma concentrations of lipid and lipoprotein fractions are related to the development and progression of various diseases, such as cardiovascular disease (CVD) and acute myocardial infarction (AMI) [4, 5]. Many studies on humans and animals have demonstrated the ability of dietary fiber such as lignin to reduce cholesterol levels by binding to bile acids in the intestine [6, 7]. The purpose of the present review is to highlight recent findings related to the chemical structure, the properties,

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and the effects of dietary fibers mainly lignin in controlling hyperlipidemia and their effects on gut microbiota.

2. METHODOLOGY

The information in this review paper was collected from 113 eligible papers that appeared on Google Scholar, Research Gate, and Scopus after searching on lignin, biopolymers, gut microbiota, and hyperlipidemia terms.

3. DIETARY FIBERS

3.1. Biopolymers

The definition of dietary fibers has been changed by the scientific community throughout the decades. Dietary fibers are comprised of the remnants of plant material that resist digestion by human gastrointestinal enzymes. The definition includes all indigestible polysaccharides such as celluloses, hemicelluloses, oligosaccharides, pectin, gums, waxes and lignin [1, 2]. Dietary fibers are categorized into two groups depending on their solubility in water; the first group is the insoluble fibers that are abundant in lignin, cellulose, and hemicelluloses, and the second group is the soluble fibers like pectin, guar gums, oat bran, and psyllium [3].

The irregular properties of polymer surfaces have been utilized since ancient times [4]. In fact, unique interfacial dynamics and high porosity of polysaccharides make them feasible candidates for pharmaceutical lipid adsorption applications [4, 5]. Pectin has been extensively evaluated for oil capsulation capacity in the digestive tract multiple times and after a multitude of clinical trials, was approved by the EFSA for the treatment of hypercholesterolemia and obesity [4]. The positive effect of dietary fibers on human's cholesterol levels and oil absorption/adsorption has been reported in numerous studies [6-10]; however, the individual effect and contribution of the lignin to this effect have been practically neglected so far.

3.2. Lignin

3.2.1. Lignin as the Necessary Polyphenolic Polymer in the Plant Structure

Lignin is the second most abundant natural polymer, the largest source of polyphenols and phenolic compounds, and the second largest biopolymer available on Earth, following cellulose [11, 12], being present in plant biomass composition in about 20–35% [13]. The term "lignin" comes from the Latin word 'lignum', which means wood [14]. Many kinds of wood constitute different percentages of lignin for example, the fraction of lignin varies widely between hardwood 32% and softwood 25%.

Lignin has a molecular weight that ranges between 700 and 100.000 Da and has a complex structure because of aromatic alcohols that are derived from phenylpropanoids [15]. These alcohols or monolignols are represented as H (p-hydroxyphenyl), G (guaiacyl), and S (syringyl) [15]. However, aromatic rings consist of different numbers of the methoxy groups [15]. It is characterized by complex cross-linked phenyl propane polymer, dark-colored, hydrophobic, insoluble in water and 72% sulfuric acid but soluble in both strong concentrated acids and concentrated bases [15-20].

Lignin is responsible for providing rigidity to the plant cell wall through the lignification process [21], the process includes holding the cellulose and hemicellulose fiber together which allows plants to expand significantly in size and height [15, 16]. Lignification also protects plant cells against environmental stress conditions by inhibiting the enzymatic hydrolysis of microorganisms and enhancing water movement in plants [16, 22-24].

3.2.2. Lignin extraction challenges and as its industrial by-product

It is almost difficult to extract lignin from plant material because of its complex structure that holds cellulose and hemicellulose together. Even the typical isolation methods such as alkali extraction, extensive milling, or enzymatic isolation can't achieve typical degradation, high purity, or less contamination of lignin [17]. Nowadays, technical lignin (also called native lignin) can be generated as a by-product of the industrial process (e.g., cellulose pulp from paper production and lignocellulose biomass from ethanol production).

Industries that use plants as a starting material for their processes generate an enormous amount of waste products and lignin is considered one of these bio-based waste materials, the reason for this, is that the major components of interest are still cellulose and hemicellulose, as these biomolecules have a clear application pathway [16, 25]. The industrial-scale production of technical lignin as the scum of the pulp and paper industry occurs after the delignification process, being sulfite, kraft, and soda [26, 27]. Technical lignin can also be obtained from emerging biorefining processes, including organosolv (OS), steam explosion (SE), ammonia fiber expansion (AFEX), and hydrothermal pretreatment (AS) [26, 28-31]. Unsurprisingly, the chemical, physical, and technical properties of these lignins strongly depend on their origin, extraction, and purification processes. The products of these methods will differ based on the type of linkage that every process aims to cleave. For instance, harsh acidic and alkaline conditions will result in the splitting of C-O bonds that interrelate the monolignols, cleavage of lignin-carbohydrate linkage, and sulphonation of the lignin aliphatic chain [26, 28-31].

3.2.3. A Novel Technique to produce Highly Pure Lignin

A number of novel biorefining technologies focus on the separation and valorization of the three main biomass components: lignin, cellulose, and hemicellulose; considering that lignin is no longer a residue but a source of potential added value [29, 30]. With today´s society's demand for environmentally friendly processes and products, hydrothermal pretreatment (AS) and hydrothermal processing (HP) can be considered promising methods to obtain high-value lignin, which ideally should be sulfur-free, solvent-free, and sustainable. A highly pure lignin can be produced using the OS process in, which it is separated from the biomass using organic solvent (mostly ethanol or methanol) at high temperatures (100-250°C) and pressure. OS lignin is soluble in organic solvents, has low molecular weights, and contains insignificant amounts of carbohydrates and ash [32, 33]. Aquasolv lignin is separated from the biomass by treatment with compressed water under a pressure ranging from 30 to 50 bar, at 180–230 °C for 10–40 min [34-36].

3.2.4. Lignin in the Commercial and Medical Industries

Lignin has found its way in producing certain economic value for biorefineries, where it is mainly used for energy production and the literature shows promising results for lignin to be applied in chemical synthesis and plastic and material applications, however, the efforts to valorize this material into goods and to bring these applications to the market are yet to be improved. Several technical lignins (kraft, soda, lignosulfonates) are already being applied in low-value applications such as cement admixtures and viscosity modifiers [32, 33]. In addition, it can be found in dyes, paints, emulsifiers, binding, thermosets, synthetic floorings, and sequestering and dispersal agents. OS lignin can be used in formulations of resins (i.e., epoxy and phenol-formaldehyde), stabilizers, and filler in polyurethane foams, adhesives, and dispersants with biocidal properties [32, 33]. Aquasolv has been used in aerogels for biomedical applications, antioxidant filler in adhesives for human consumption, and alternative to activated charcoal [34-36]. Lignin offers the stability and mechanical impact as an active filler for natural rubber [20]. However, due to the natural properties that lignin exerts in nature, the possibility to enter high-value markets where biobased materials are required is endless, for instance, lignin can be used in cosmetic formulations, as a natural ingredient and it can be used as antioxidant, antifungal, antiparasitic and anticarcinogenic activity [16]. Furthermore, lignin produced by novel bio-refinery processes such as OS is known to be non-cytotoxic, cheap to produce, and has no interaction with the human intestine due to its branched fractions. This has led to the evaluation of such lignins as potential oil adsorbents and, consequentially, anti-hypercholesterolemic agents. They can be used against

hypercholesterolemia and obesity, also to decrease the incidence of chronic degenerative disease [25]. Generally, near-nature structured lignins are reported as indigestible biopolymers, however, some authors report that small parts can be broken down and fermented in the small intestine. Others report that only 10 % of lignin is mainly digested in the stomach and the rest is indigestible and is excreted completely in the stool [19]. Many studies reported that lignin can adsorb hydrocarbons and toxins [18, 19], and resist bacterial disintegration in the gastrointestinal tract more than any other natural polymers [15, 16].

3.2.5. Nano-sized Lignin Applications and Benefits

A recent study has reported that the incorporation of OS lignin into nanoscale zero-valent iron boosts its hexavalent chromium detoxification performance in the aquatic environment [37]. In recent years, lignin has gained considerable attention in synthesizing nanoparticles for manufacturing lignin-based nanomaterials to take advantage of unexplored lignin in high-value-added applications. For example, nano-sized lignin has added numerous values to industrial products by improving the durability of items such as rubber and textiles [38]. Also, enhanced UV shielding, besides, the quality, chemical and physical properties. Furthermore, lignin nanosized has a wide application in sterile biomedical devices, food packing materials, cosmetics industrial and tissue engineering [38, 39]. Lignin is the most suitable choice in medical research. According to the biodegradability, absorption capacity, non-toxic properties, antibacterial, antioxidant, and anti-parasite activity, lignin nanoparticles with various dosage forms have significant potential for drug delivery [40, 41]. Lignin nanoparticles as drug delivery are capable of loading hydrophobic drugs such as coumarin-6 and hexadecane, and hydrophilic drugs such as rhodamine 6G and doxorubicin hydrochloride (DOX) for cancer treatment. [39]. A recent study has evaluated the efficacy of the anticancer drug doxorubicin hydrochloride (DOX) and demonstrated the higher efficacy of DOX-loaded folic magnetic-functionalized lignin nanoparticles. In another study, when lignin was combined with microcrystalline cellulose as an excipient in tablets, the results showed a significantly enhanced tetracycline release. Also, aspirin tablets including lignin showed a higher release rate of the active ingredient compared to the tablets without [40].

4. Lignin and hyperlipidemia

4.1. The Potential Effects of Natural Polymers on Lowering Cholesterol Level

In general, natural polymers are known to decrease the risk of coronary heart disease by lowering cholesterol level in the blood, mainly due to their physical properties, such as dispersibility in water, viscosity, binding ability, absorptive and gelling capacity as well as fecal bulking capacity [42, 43]. Furthermore**,** soluble and insoluble fibers increase the movement of food through the intestine. These are associated with a high water-holding capacity, which results in an increased viscosity in the gastrointestinal tract and promoted the binding ability of bile acids in the small intestine. This consequently leads to reduce the absorption of cholesterol by bile acids and lowers its blood levels [44]. Another benefit is that soluble fibers interfere with sugar absorption to reduce the blood sugar concentration and provide short-chain fatty acids as by-products of fermentation in the colon. While insoluble fibers produce bulk effects on feces and the fermentation process occurs in the large intestine [6]. The gelling capacity of some biopolymers is also used to increase the sensation of satiety in consumers, resulting in lowering ingestion of potentially high-fat diets. Due to these properties, natural polymers such as chitosan, pectin, guar gum, and lignin are considered to be interesting candidates for the treatment of lipids high blood levels.

4.2. Overview of Hyperlipidemia

Hyperlipidemia is a disorder of lipid metabolism characterized by excessive accumulation of one or more of the lipids in the plasma, such as high serum cholesterol level in the bloodstream (>240 mg/dl) [45, 46]. Also, it can

be defined as a lipoprotein metabolic disorder associated with high (>159 mg/dl) serum low-density lipoprotein cholesterol (LDL-C) [47]. Hyperlipidemia is one of the most dangerous risk factors for cardiovascular disease (CVD), such as coronary heart disease (CHD) atherosclerosis, and stroke [48, 49]. A study reported that 12 million people die because of cardiovascular disease each year worldwide and according to the World Health Organization (WHO), by 2030, CVD will affect approximately 23.6 million people globally [49-51].

Published studies reported an inverse correlation between the low blood level of high-density lipoprotein cholesterol (HDL-C) (which is below 40 mg/dl) and coronary heart disease. [52, 53]. Accumulation of cholesterol and other lipids in the atrial wall is the major cause of atherosclerosis, which is considered a key complication of cardiovascular diseases, such as acute infarction and hypertension [50, 54]. Lifestyle, dietary, and genetic disorders (e.g., familial hypercholesterolemia) are recognized as contributory risk elements for the development of hypercholesterolemia. Clinical trials demonstrated that a reduction in low-density lipoprotein cholesterol (LDL-C) levels could lower the incidence of atherosclerosis and CVD [46]. Another study indicated that a 1 % reduction in cholesterol level could decrease the risk of coronary heart disease by 2-3 % [51, 55].

4.3. Statins medications: Action Mechanisms and Side Effects

Statins medications -which are hydroxy-methyl-glutaryl-coenzyme-A (HMGCoA) reductase inhibitors- are the most widely used for the treatment of hyperlipidemia and cardiovascular disease (CVD) in many countries [56, 57]. They target hepatocytes and prevent HMG-CoA reductase enzyme that converts HMG-CoA into mevalonate, mevalonic acid is a precursor for cholesterol and non-steroidal compounds, therefore, the inhibition of mevalonate production reduces the level of cholesterol. Other action mechanisms of statins include: the improved uptake and degradation of low-density lipoproteins-cholesterol (LDL-C), prevention of the lipoproteins, and inhibition of the scavenger receptors expression [58]. Statins are efficient in the treatment of that condition; however, their intake has been positively correlated with various adverse effects like liver failure and muscle pain, which may lead to dose reduction or discontinuation of the treatment [57]. The most important side effect is muscle symptoms which reported about 25% of symptoms, including mild myalgia that may affect around 10% of statin users [57]. Another side effect is liver toxicity, generally, 2 - 3% of reported cases stated an increase in the serum liver enzymes [59], and few reports are associated with liver failure among statin users [57].

Due to the negative side effects of statins and the overall negative perception by the population in use, many research and development activities focus on finding new methods to control hyperlipidemia such as dietary intervention and natural/herbal products (e.g., biopolymers) [47]. This is because, the current hypolipidemic medicines are expensive and have serious side effects and the natural products show multi-action activity among various medical conditions, such as, CVD, inflammatory disease, and cancer [49, 51].

4.4. Lignin as Alternative Choice to Manage Hyperlipidemia

Diets rich in fiber were found to lower the total blood cholesterol levels by 26% plus LDL-C levels by 29% in normal and hyperlipidemic adults [60]. Moreover, studies have shown a decreased risk of hyperlipidemia when consuming food rich in antioxidants and biopolymers. Lignin is one of the biopolymers with the most potential effect to decrease cholesterol level, not only due to their functionality and abundance but also due to its low price [4, 7-9].

Generally, the lignin effect and chemical composition depend on several factors such as its plant source, the extraction process, and biomass used. A new oral formulation which includes lignin admixed with methylcellulose was investigated for hyperlipidemia

treatment. The combination with methylcellulose facilitates lignin dispersion in the gastrointestinal tract. Therefore, treating hyperlipidemia patients by administering lignin results in about a 20% reduction in blood cholesterol levels. Lignophenols (LPs) are high-purity lignin derivatives with ahigh phenolic content, and are described as stable molecules, although their physiological role is still unclear. Treatment with LPs markedly decreased oleate-induced apo-B secretion from HepG2 cells in a dose-dependent manner [25]. In general, dietary fibers have exhibited several actions on the intestine by increasing peristaltic movement, stimulating intestinal food passage, increasing feces volume andweight, and transit time [43]. Researchers studied the impacts of the indigestible residue of foods on the bulk of the stool, especially the impacts of cellulose, hemicellulose, and lignin on laxation. By analysis of the stool, it was found that lignin is digested faster than cellulose and hemicellulose and it was found in high percentages in the residue that recovered in the stool, while cellulose and hemicellulose went through the human gut in smaller amounts. Therefore, the number of residues disappearing from the gut influences the volume of the stool more than the number of residues fed or the amount recovered in the stool [61].

For over five years, Hillman and his coworkers [62] focused on the dietary fiber components and their various actions on the human body. They conducted many studies to determine the impact of daily dietary supplementation with pure pharmaceutical citrus pectin 12 g/d, pure alpha-cellulose 15 g/d, and pure auto-hydrolysis lignin 12 g/d on the human body. It was found that cellulose decreased stool pH from 6.38 to 6.12, decreased stool pass time by 27%, and increased in stool weight by 57%. In comparison with pectin and lignin, cellulose showed a significant change in stool characteristics while pectin and lignin did not. On the other side, neither cellulose, pectin, and hydrolysis lignin significantly changed serum cholesterol levels in healthy subjects over eight weeks [63]. However, a study was conducted in diet-induced obese mice, while using a lignin-rich fraction of brewer's spent grain. In the animal model, body weight gain was significantly reduced with all fibers tested, but only the lignin-rich fraction and lignin-rich fraction cellulose decreased fasting plasma low-density lipoprotein cholesterol and total cholesterol compared to the high-fat diet group. The results suggest that the consumption of lignin-rich fraction induced beneficial systemic changes in mice via gut microbiota, bile acids, and gene expression in the liver. More studies in the future should focus on through assessments of the effect of different types of lignin on the organism's physiology and targeted physiological effects. The establishment of a structure-function relationship is crucial to unravel the true potential of lignin for high-value applications like the case of food, nutraceuticals, and pharmaceuticals.

5. MISCELLANEOUS BIOPOLYMERS AND TREATMENT OF HYPERLIPIDEMIA

Polymers synthesized by living organisms are considered to be interesting candidates for industry and pharmaceutical lipid adsorption.

5.1. Chitosan in the Previous Studies

Chitosan is a natural polymer that has been evaluated for oil capsulation proficiency in the digestive tract, and it is available in the market as a counter (OTC) drug for fat adsorbents and hypercholesterolemia treatment [19, 64].

5.2. Cellulose in the Previous Studies

Another example of a biopolymer that is used to improve lipid profile is cellulose, a polysaccharide that is the most prominent component of plant cell walls [6]. The effect of cellulose didn't exert any significant change on serum lipids of high-fat diet rats models for six weeks [65]. On the other hand, the effect of cellulose was examined in healthy volunteers, after three weeks of treatment with cellulose, there was no significant change in serum cholesterol levels but the weight of the feces was changed [66]. In other studies, doses of 15-20 g/dL of cellulose

were given to adult volunteers for four weeks and it was observed that a small increase in stool output can be achieved [66]. Evaluation of hypolipidemic impacts of dietary insoluble fibers has been extensively investigated in mice, for instance, a study in which mice were fed a high-fat diet over three weeks found that serum cholesterol levels can be decreased when using three types of fiber (cholestyramine, chitosan, and cellulose). In particular, cholestyramine, which resulted in decreased lipid absorption and increased fecal bile acid output. In contrast, cellulose did not affect cholesterol absorption or fat excretion in mice [67]. In a similar study, the effect of cellulose on lipid metabolism in rats was compared with that of other soluble dietary fibers such as pectin and guar gum. Cellulose-feeding mice showed the highest blood cholesterol levels and body weight gain compared to pectin and guar gum-feeding mice. Moreover, pectin and guar gum with high molecular weight were more effective in fecal fat excretion and bile acids than cellulose [44]. The viscosity of the guar gum in the stomach is one of the important reasons for its ability to reduce total cholesterol in humans and animal models. The high viscosity can influence the absorption and the digestion of food. Guar gum has been found to prolong the retention of chyme in the intestine slowing down the food digestion process, thus, resulting in lower total cholesterol levels. The mechanisms of action for pectin have been well-studied in the literature.

5.3. Pectins in the Previous Studies

Pectins are natural polymers composed of groups of polysaccharides and are mainly obtained from fruits and vegetables. Currently, they are commercially extracted (citrus peel or apple pomades) by chemical or enzymatic methods [68]. The mucilaginous fibers such as pectin, guar gum, and oat bran form gel in the small intestine which interferes with the absorption of total cholesterol and bile acids [69]. Different gelling fibers might achieve approximately a 7% reduction in total cholesterol levels. Pectin showed an effective decrease in plasma cholesterol levels and liver cholesterol synthesis among animal models such as rats, hamsters, chickens, and rabbits, where dosages of 6-15 g/day for 4 weeks [70]. Many studies conducted on rats and healthy adults recommended pectin with a high methoxyl group as an effective hypo-cholesterolemic agent [71, 72]. Thereafter, low-methoxyl pectin presented the most hypolipidemic effects when the low-fat diet was given to rats, but regarding the high-fat diet, pectin had no impact on serum lipids [65, 73]. Although, using high methoxyl pectin in young healthy adults showed a significant alleviation in blood cholesterol, there was no difference in using high methoxyl pectin on fecal lipids compared to pectin with less methoxyl content [71]. This result could be understood from the capacity of pectin in forming gel, which is required in lowering the cholesterol levels rather than differences in bile acid binding due to different methoxyl contents [71].

5.4. Guar Gum in the Previous Studies

Guar gum is derived from the endosperm of *Cyamopsis tetragonoloba*. Chemically, guar gum is a mixture of polysaccharide consisted of galactose and mannose [68]. It forms a highly viscous gel when dissolved in water. Studies showed that guar gum has a strong influence on reducing cholesterol levels and body weight in hyperlipidemic rats [43]. Also, the impact of guar gum on healthy rats was investigated for 28 days, the results revealed that guar gum markedly decreased total cholesterol levels (TC), triglyceride (TG), low-density lipoprotein (LDL) as well as high-density lipoprotein (HDL). These results concluded that guar gum had hypolipidemic effects in both hyperlipidemic and normal rats which may prevent atherosclerosis [74]. The effect of guar gum was also studied in patients with primary hyperlipidemia over six weeks. The results showed that the blood cholesterol and LDL levels decreased as compared to the control group. Clinically, guar gum has been used in treating patients with type two hyperlipidemia to reduce total cholesterol levels [69, 75]. It was noticed that the

stool volume and output frequency in humans given partially hydrolyzed guar gum were high, which might be related to the ability of guar gum to lower serum cholesterol levels by increasing cholesterol elimination as fecal bile acids [75-77].

5.5. Psyllium in the Previous Studies

Psyllium is derived from the psyllium seed husk (PSH) of *Plantago ovate.* Studies on PSH showed that its gel-forming carbohydrate is not able to be fermented, this non-fermentation is responsible for the laxative and hypolipidemic activity [78]. This laxative effect alleviates constipation through absorbing water from the gastrointestinal contents and consequently increasing stool volume [78]. As a hypolipidemic, the impact of psyllium was studied in subjects that were administrated 9.6 g of psyllium per day into their usual diets for over five weeks. The results showed that there was a decrease in blood lipid levels by 9% [60]. Another study demonstrated a reduction in total cholesterol and LDL concentration of about 5-17%, and 8-20% respectively. This reduction was observed in mild to moderate hypercholesterolemic patients when psyllium was incorporated as 3.4–10.2 g three times daily for 12 weeks [60, 69]. Moreover, a meta-analysis study was performed on hypercholesterolemia patients consuming psyllium-enriched cereal as a part of the low-fat diet, results also showed lowered cholesterol levels and LDL concentrations [6].

5.6. Oat and Wheat Bran Fibers in the Previous Studies

Oat bran dietary fiber has a lower density than wheat bran dietary fiber, it contains 50% of water-soluble fiber, whereas wheat bran contains 8% of water-soluble fiber. However, oat and wheat brans influence lipid metabolism differently. A study that evaluates the impacts of oat and wheat brans on lipid profile in healthy adults showed that oat bran significantly lowers cholesterol and LDL blood levels, but, no significant change was noted with wheat bran [79]. In an *in vivo* study, wheat bran demonstrated no hypolipidemic effect in rats fed on both the low and high-fat diet but showed decreased VLDL and lowered serum TG with the high-fat diet [65]. *Borel et. al* (1989) reported in their study that wheat bran had no impact on human lipid profile whereas in rats it decreases total cholesterol and triglyceride in the liver [80]. In another study, the effects of oat and wheat brans on human large bowel were determined. The results showed that oat bran increases fat excretion and stool weight by fermentation of soluble fiber in the colon [81]. In hyperlipidemic men, oat bran demonstrated a reduction in total cholesterol levels through decreasing bile acid and cholesterol absorption and stimulating fecal bile acid excretion [82].

6. GUT MICROBIOTA

Microbes live on all human body surfaces, but a significant number of these microbes habitat in the gastrointestinal tract. The human gut contains more than 10000 microbial species that form a complex ecological community called gut microbiota. Bacteria are classified into aerobic and anaerobic organisms, the latter is the most bountiful of gut microbiota such as members of the phyla *Firmicutes*, *Bacteroidetes*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, and *Peptostreptococcus*. The main facultative anaerobic bacteria are *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, and *Proteus* [83, 84]. The gut microbiota has important benefits on host metabolism, immune system, vitamin synthesis, protection against different diseases, improving angiogenesis, and regulation of lipid storage. For example, some bacteria ferment the undigested food to produce short-chain fatty acids such as acetic, propionic, and butyric acid which are rapidly absorbed from the colonic mucosa to supply energy to the epithelial cells [85]. Due to this, the gut microbiota has lately been defined as a "vital organ" because of its multi-interactional with other organs through several pathways such as the immunological, and metabolic paths. Any imbalance in the gut microbial diversity not only leads to gut-related problems but also affects other organ-related diseases such as gastrointestinal disease, obesity,

colorectal cancer, and hyperlipidemia [83, 84]. Gut microbiota diversity depends on several host factors such as diet, age, and environmental factors [86]. However, diet is now considered one of the main factors in changing the gut microbiota; thus, this manipulation of the microbiota could be used as a therapeutic approach to inhibit or treat several diseases. Diet, probiotics, antimicrobials, and fecal microbiota transplant are considered potential strategies for microbiota manipulation [84].

6.1. Lignin and gut microbiota

The resistance of lignin to breakdown both in the environment and the gastrointestinal tract is higher than cellulose and hemicellulose. For this reason, lignin is one of the materials that is harder to valorize as the conditions for depolymerization and further use are expensive and use harsh chemical conditions. Bacteria are known to degrade lignin belong to actinomycetes, some Firmicutes, α-proteobacteria, and γ-proteobacteria [23]. These are responsible for lignin biodegradation through producing three enzymes, lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccases. These enzymes are responsible for the breakdown of lignin bonds by depolymerization processes, such as catalytic reduction, hydrolysis reaction, and catalytic oxidation [14, 23]. The expression of these enzymes differs based on the microorganism, many strains with the best-known degrading ability of lignin were identified in γ – proteobacteria, specifically, *Pseudomonas fluorescens* (produce lignin peroxidase), *Pseudomonas putida* (produce manganese peroxidase), and *Escherichia coli* (produce laccase). Moreover, lignin-degrading enzymes were identified in wood-degrading fungi, which mostly live as saprotrophs or parasites in ecosystems [14, 23]. All types of fungi, particularly white-rot fungi can degrade lignin totally to CO_2 and H_2O. For example, *Marasmius quercophilus* producing laccase, and *Agaricus bisporus* producing laccase and manganese peroxidase [14, 23, 87].

6.1.1 lignin degradation by human gut microbiota

In general, maintaining a good and healthy gut microbiota plays a key role in our health, gut microbiota helps control the digestive and benefit the immune system, thus having an impact on several health aspects. An imbalance or unhealthy population of microbes in the intestine can significantly contribute to weight gain, high blood sugar, and high cholesterol as well as other disorders. The effect of lignin, especially alkali lignin from brewer's spent grain, was studied on the gut microbiota [88]. The soluble fraction of lignin obtained after enzymatic hydrolysis was found to be altered in the *in vitro* colon fermentation. Conversely, the insoluble lignin fraction remained unaltered. Another important finding of this study is that none of the fractions evaluated inhibited the growth of *Lactobacillus* and *Bifidobacterium* bacteria, concluding this as a very positive outcome [88]. For the soluble fraction of the alkali lignin, if metabolites are formed during its fermentation, it will be important to assess the health- effects of these, thus opening new research fields and applications for lignins.

6.1.2 lignin degradation by animals' gut microbiota

A very limited range of organisms can decompose lignin more than cellulose. For example, some bacteria and white-rot basidiomycetes are considered as the primary degraders of lignin by producing oxidative enzymes [89]. Terrestrial animals such as termites use lignin as a nutrient resource. The role of gut microbiota in cellulose degradation in termites has been well documented, but lignin degradation in termites remains unclear. Several bacteria have been isolated from termites that exhibit degradation capabilities of lignin such as *Acinetobacter calcoaceticus* from *Mastotermes darwiniensis* termite, *Bacillus firrnus* from *Reticulitermes santonensis*, *Comamonas acidotvorans* from *Nasutitermes nigriceps*, and *Rhodococus erythropolis* [90-92]. Moreover, the degradation of lignin and lignin-derived aromatic compounds by the gut microbes was studied in termites-feeding guilds such as wood, soil feeders, and fungus cultivators [91]. Recent results demonstrated that bacterial degradation of lignin in the guts of wood-feeding termites such as *Nasutitermes lujae* and *Reticulitermes flavipes* have a profound impact on aromatic

compounds only in the presence of oxygen [91].

Ruminant animals consume lignin-including grasses but can only digest the polysaccharides, this happens with the microbial biodegradation of cellulose and hemicellulose in the rumens [17]. Ruminants can exploit fiber components such as lignin efficiently because their digestive system depends on microbial degradation in the stomach [93]. However, the microbial activity in the rumen showed a potential breakdown of benzyl ether bonds of lignin under anaerobic conditions. [94]. It has been found that fungi are capable of degrading the lignocellulose complex to its fractions e.g. cellulose, hemicellulose, and lignin. Twelve species of Cyathus were isolated from cattle dung to evaluate lignin biodegradation in kenaf by fermentation of the sum of ^{14}C released into solution and ^{14}C released into the gas phase over a month. Three species were able to remove lignin more rapidly than other species e.g. *Cyathus pallidus*, *C. africanus*, and *C. berkeleyanus*. While *Cyathus canna* has particularly the greatest degradation observed of lignin than other plant parts [95]. In wheat straw, *Cyathus stercoreus* showed great lignin degradation as matched with cellulose [96]. The extraction of lignin was using alkaline media, and the samples were taken from cow dung and other sources. The most collective positive results came from *Pseudomonas sp* which acts as a lignin-degrading bacteria by oxidative enzymes [97]. Further studies on the giant panda discovered that it lacks lignin-degrading enzymes, which are responsible for lignocellulose digestion. It was proven that *Pseudomonas putida* and the mangrove forest bacteria were integrated with lignin degradation by laccase enzyme. The microbial gut flora of giant panda indicated that lignolytic enzymes may facilitate lignin breakdown [98].

6.2. The effect of other biopolymers on gut microbiota

6.2.1 Degradation of cellulose and hemicellulose by gut microbiota

Although only soluble fibers can be fermented by colonic microbiota insoluble are poorly fermented by gut microbiota [99], the effect of insoluble fibers on gut microbiota was investigated. In a recent paper by *Kim et al.* (2020), it was found that small amounts of cellulose are fermented by mammalian gut microbe while the rest is poorly fermented by non-mammalian gut microbes [100]. Contrary to giant pandas, degradation of cellulose inside the rumen has been associated with bacterial phyla *Firmicutes* and *Fibrobacteres* [101], because the composition of their digestive system lacks the necessary enzymes, which are responsible for cellulose digestion [102]. One of the studies suggested a new alternative method for cellulose degradation by utilizing rumen *Bacteroidetes* and polysaccharide utilization locus-catalyzed conversion of cellulose [101]. The beneficial role of cellulose has been performed on the gut microbiota of mice for 3 months. *Akkermansia, Parabacteroides, Lactobacillus, Clostridium, Eisenbergiella, Marvinnbryantia,* and *Romboutsia* were moer abundant in a high cellulose diet than a low cellulose diet, these findings indicated that cellulose can play a prominent role in maintaining gut hemostasis [100]. Effects of cellulose on the gut microbiota of aquatic animals have been studied on Atlantic salmon, only *Staphylococcus equorum* reported high production of cellulase enzyme [103].

6.2.2 Degradation of pectin by gut microbiota

One of the studies on dietary fiber like pectin detected that the levels of *Lachnospira, Dorea, Clostridium,* and *Sutterella* were increased after pectin fermentation by the human fecal microbiome, as well as increased in short-chain fatty acids (acetate and butyrate) levels after incubation with pectin [104]. Different sources of pectin also modulated human fecal microbiota, for instance, soy pectin showed a positive impact on *Lactobacillus rumis* [99]. In rats, fecal microbiota composition was assessed before and after citrus pectin supplementation, which led to an increases in the members of *Prevotellaceae* and *Ruminococcaceae*with no considerable changes were associated with pectin supplement in the gut microbiota [105]. In comparison with high-fat diet, apple-derived pectin has restored the normal levels of *Bacteroides* and

Lactococcus in the gut microbiota of rats [106].

6.2.3 Degradation of Guar gum and psyllium by gut microbiota

Guar gum is considered one of the beneficial soluble fibers on gut microbiota. In an *in vivo* study, the intake of partially hydrolyzed guar gum increased the levels of fecal beneficial bacteria *Bifidobacterium* and *Lactobacillus* [85]. In another *in vivo* study, it has been reported that short-chain fatty acids such as butyric acid are stimulated by fecal fermentation of partially hydrolyzed guar gum [85]. Furthermore, the impacts of hydrolyzed guar gum on gut microbiota and bowel movements revealed that genus levels of *Bifidobacterium*, *Ruminococcus*, and *Megasphaera* were increased, and the bowel movements were improved by consumption of hydrolyzed guar gum [107]. Additionally, guar gum has proven to prevent inflammatory bowel disease such as colitis in mice by adjusting gut microbiota [108]. Moreover, guar gum increased the abundance of *Bifidobacterium* in Wister rats using different viscosity preparations [99].

One of the recent studies investigated the effect of psyllium on constipation and gut environmentand showed that psyllium supplementation to healthy volunteers had significantly increased *Veillonella* and decreased *Subdoligranulum* [109]. In contrast, in constipated patients, the abundance of *Lachnospira*, *Faecalibacterium*, *Phascolarctobacterium*, *Veillonella*, and *Sutterella* was increased, and the levels of acetate and propionate were different [109].

Recently, researchers have been paying attention to cereal fibers and their activity on gut microbiota. The majorities of research have explored the potential effect of wheat bran on the gut environment on healthy adults.*Bacteroidetes*, *Firmicutes*, and *Actinobacteria* increased after the consumption of wheat bran [110]. Furthermore, one of the studies reported an increase in *lactobacilli* and *bifidobacteria* in total fecal bacteria levels after taking wholegrain oat granola by hyperglycemic or hypercholesterolemic patients [110].

Table 1. Quick Information in Brief

Dietary Fibers	Biological Mechanisms	Biological Effect on Lipid Profile	Biological Effect on Gut Microbiota
Cellulose	-Increases bowel movements and fecal output [66]. -Decreases stool pH [62].	-No significant effect on serum lipids [65].	-Increases beneficial bacteria like *Akkermansia, Parabacteroides, Lactobacillus, Clostridium, Eisenbergiella, Marvinnbryantia,* and *Romboutsia* [100].
Pectin	-Forms gel in the small intestine which interferes with the absorption of total cholesterol and bile acids [69]. - The gelling capacity increases the sensation of satiety in consumers and reduces the ingestion of high-fat diets [6].	-Lowers total cholesterol and liver cholesterol synthesis [70].	-Increases beneficial bacteria like *Lachnospira, Dorea, Clostridium*, and *Sutterella* [104]. -Boosts SCFA production (acetate, butyrate) [104].
Guar Gum	-Increases the stool volume and output which leads to eliminating the cholesterol as fecal bile acids [75-77]. - The gelling capacity increases the sensation of satiety in consumers and reduces the ingestion of high-fat diets [6].	-Decreases total cholesterol levels (TC), triglyceride (TG), low-density lipoprotein (LDL), and body weight [43].	-Increases beneficial bacteria like *Bifidobacterium, Lactobacillus*; *Ruminococcus,* and *Megasphaera* [85].

Dietary Fibers	Biological Mechanisms	Biological Effect on Lipid Profile	Biological Effect on Gut Microbiota
Psyllium	-The laxative effect alleviates constipation by absorbing water from the gastrointestinal contents and consequently increasing stool volume [78].	-Lowers cholesterol levels and LDL concentrations [6].	-Increases beneficial bacteria (*Veillonella)* and decreased harmful bacteria (*Subdoligranulum)* [109]
Oat Bran	- Increases fat excretion and stool weight [81]. -Decreases bile acid and cholesterol absorption [82]. -Stimulates fecal bile acid excretion [82].	- Significantly lowers cholesterol and LDL levels [79].	-Increases beneficial bacteria (*lactobacilli* and *bifidobacteria)* [110][110].
Wheat Bran	-Increases the movement of food through the intestine [44].	-No impact on human lipid profile [79].	-Increases beneficial bacteria (*Bacteroidetes*, *Firmicutes*, and *Actinobacteria)* [110]
Chitosan	- The gelling capacity increases the sensation of satiety in consumers and reduces the ingestion of high-fat diets [6]. -Decreases the absorption of dietary fats [19, 64].	- Decreases cholesterol level [6].	
Lignin	Binds to bile acids in the intestine [6, 7]. - Increases peristaltic movement, stimulats intestinal food passage, increase feces volume and weight, and transit time [43]. - The gelling capacity increases the sensation of satiety in consumers and reduces the ingestion of high-fat diets [6].	- Decreases cholesterol levels [4, 7-9].	-Increases beneficial bacteria (*Lactobacillus* and *Bifidobacteri)* [88].

7. CONCLUSION

It is obvious that each polymer has a unique feature or characterization that allows it to influence hyperlipidemia and the gut microbiota of both animals and humans. The type of dietary fiber whether soluble or insoluble (lignin, cellulose pectin, or guar gum, etc.) associated with different mechanisms of action, has demonstrated beneficial effects on high cholesterol levels in the blood accounting in several studies that are included in this review. Furthermore, many studies revealed that biopolymers could be used not only in prevention but also in the treatment of hyperlipidemia and its related complications. In addition, positive impacts were reported on the normal bacterial community by consuming biopolymers. Different biopolymers can support different useful groups of gut microbiota; thus, protecting the health of the gut. Taken together, it has been demonstrated that over the years the possibilities of using biopolymers in many applications aspects in the research fields have increased. The physicochemical and structural diversification of biopolymers such gel-forming, texturing, thickening, interfacial adsorption ability, and health-associated properties give them the potential use in the food and nutraceutical industry which is expected to

grow over the next years. But so far, there are limited studies on the bright aspects of dietary fibers, specifically lignin. Future research is still needed to investigate the effectiveness of lignin when combined with current anti-hyperlipidemia drugs, possibly using nanoparticle technology, for example.

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تأثير اللجنين والبوليمرات الحيوية الأخرى على فرط شحميات الدم وميكروبات الأمعاء

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ملخص

حتى الآن، دُرست الألياف الغذائية، مثل اللجنين والسليلوز والبكتين وصمغ الغوار والسيليوم، بشكلٍ مُكثّف لإمكاناتها الوقائية والعلاجية باستخدام نماذج حيوانية وبشرية، وخاصةً لتأثيراتها الإيجابية على الحالات الأيضية المزمنة، مثل اضطراب شحميات الدم والاضطرابات المُرتبطة به. يُعدّ اضطراب شحميات الدم اضطرابًا أيضيًا خطيرًا يرتبط بارتفاع كوليسترول الدم، ومرض الشريان التاجي، وأمراض القلب التاجية. أظهرت أبحاث سابقة أن هذه الألياف الغذائية قادرة على خفض مستويات الدهون المرتفعة في المصل من خلال آليات مختلفة. ومن أهم هذه الآليات تعديل ميكروبات الأمعاء. فزيادة بكتيريا حمض اللاكتيك (LAB)، القادرة على استقلاب أنواع مختلفة من الألياف الغذائية مثل اللجنين، قد تُخفض مستوى الكوليسترول. تهدف هذه المراجعة إلى تقديم رؤى مفيدة ومناقشات شاملة حول المعارف الحالية المتعلقة بخصائص وتأثيرات الألياف الغذائية، وخاصةً اللجنين، في التحكم بفرط شحميات الدم وتأثيراتها على ميكروبات الأمعاء. تُستخدم محركات البحث جوجل سكولار، وبوابة الأبحاث، وسكوبس لجمع البيانات باستخدام اللجنين، والبوليمرات الحيوية، وميكروبات الأمعاء، وفرط شحميات الدم كمصطلحات بحث.

الكلمات الدالة: اللجنين، البوليمرات الحيوية، ميكروبات الأمعاء، فرط شحميات الدم.

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Obesity is Associated with Increased Cardiovascular Risk and Increased Prevalence of Insulin Resistance among Apparently Healthy Young Adults

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ABSTRACT

Objective: Cardiometabolic abnormalities are still prevalent in young individuals. This research aims to investigate associations between obesity, cardiometabolic risk factors, and insulin resistance (IR) in apparently healthy young adults.

Methods: This cross-sectional study involved 70 obese and 70 age/gender matched young adults with normal body weight. Serum glucose, insulin, lipids, and homocysteine were measured. IR was determined using Homeostasis Model Assessment-IR (HOMA-IR). Systolic (SBP) and diastolic (DBP) blood pressures were measured. Other data were self-reported.

Results: Obese participants exhibited higher SBP, DBP, glucose, triglycerides (TGs), cholesterol, low-density lipoprotein (LDL), insulin, and HOMA-IR, and lower high-density lipoprotein (HDL) compared to healthy weight participants (p-values<0.01). Body mass index (BMI) was correlated with SBP, DBP, glucose, insulin, HOMA-IR, cholesterol, LDL, TGs, and was inversely correlated with HDL (p-values<0.01). HOMA-IR was correlated with SBP, DBP, cholesterol, LDL, and TGs, and was inversely correlated with HDL (p-values<0.01). Participants with IR had higher BMI, SBP, DBP, cholesterol, LDL, and TGs compared to participants with normal insulin sensitivity (p-values<0.05). Obesity was associated with increased SBP, TGs, insulin and HOMA-IR (p-values<0.05). There was no significant difference in homocysteine between groups (p-value>0.05).

Conclusion: Obesity is associated with increased cardiovascular risk and increased prevalence of IR in apparently healthy young adults. Pharmacological and behavioral interventions are urgently needed to manage increased cardiovascular risks among this age group.

Keywords: Obesity, young adults, cardiovascular risk, insulin resistance.

INTRODUCTION

Obesity is defined as an excessive or abnormal body fat accumulation that presents a risk to health.[1] Overweight and obesity are increasingly prevalent because of the modern life that encourages sedentary lifestyles and consumption of unhealthy fast food and sugar-rich drinks.[2] According to the latest statistics, the worldwide prevalence of obesity has almost tripled since 1975.[3] In Jordan, age-standardized prevalence of overweight and obesity among women was 70.6% as reported in the year 2021.[4]

In addition to its adverse socioeconomic consequences,[5] obesity represents a global health concern in all age groups as it is associated with increased risk of cardiometabolic complications.[6] It increases the risk of developing cardiovascular diseases (CVDs), insulin resistance (IR), Type 2 diabetes mellitus,[7, 8] and some

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types of cancers,[9] which are considered as leading causes of mortality and morbidity.[10] It also adversely affects mental health, musculoskeletal system, and is linked to sexual dysfunction.[11-13]

Cardiovascular diseases (CVDs) are considered as a major cause of death all over the world, nearly 20.5 million people died from CVDs in the year 2021, with a rate higher in low to middle income countries.[14] Obesity is mainly associated with increased risk of heart failure, coronary artery disease, and cerebrovascular diseases.[15] The mechanisms through which obesity increases the risk of CVDs include changes in body composition that affects hemodynamics and alters heart structure.[16-18] Accumulation of visceral fat is particularly associated with increased risk of CVDs.[19]

In addition to obesity, several modifiable risk factors contribute to the development of CVDs. These include smoking, high blood pressure, elevated low-density lipoprotein (LDL), decreased high-density lipoprotein (LDL), hypercholesterolemia, hypertriglyceridemia, sedentary lifestyle, diabetes mellitus,[20] as well as hyperhomocysteinemia.[21] While non-modifiable cardiovascular risk factors include age, gender, ethnicity, race, and genetics.[22]

While increasing numbers of studies examining the correlation of metabolic parameters and cardiovascular risks in obese individuals, such correlation in healthy young subjects is not sufficiently recognized. Additionally, growing evidence suggests prevalence of cardiovascular abnormalities in apparently healthy, and particularly young individuals. Indeed, the number of young adults with cardiovascular events is increasing, and only one out of four American young adults (18 – 44 years old) had an ideal cardiovascular health.[23] Therefore, we aimed to assess cardiometabolic risk factors among young obese adults compared to age/gender matched adults with healthy body weight. The relationship between obesity and other cardiometabolic risk factors in young adults needs to be investigated to predict susceptibility to developing cardiometabolic diseases in the future. We hypothesized that obese young adults have a higher risk of developing cardiometabolic diseases compared to subjects with healthy body weight. To achieve this, we aimed to assess the relationship between obesity and cardiovascular risk variables including lipid profile, blood pressure, smoking, homocysteine, blood glucose, and insulin. Additionally, we aimed to assess IR in the study groups and its association with obesity and other cardiometabolic risk factors.

RESULTS

Differences in cardiovascular and metabolic risk factors between obese participants and participants with healthy body weight

As shown in Table 1, obese participants exhibited significantly higher levels of BMI, SBP, DBP, fasting glucose, fasting insulin, TGs, total cholesterol, LDL, and HOMA-IR, and lower levels of HDL compared to participants with healthy body weight (p-values < 0.01). Homocysteine levels of all participants were within the normal range and there was no significant difference in homocysteine between obese participants and participants with healthy body weight (p-value $= 0.34$). In addition, there was no significant difference in smoking, marital status, education, number of family members, average family income, family history of CVDs and diabetes, and doing regular exercise between obese participants and participants with healthy body weight (p-values > 0.05).

Table 1: General characteristics and differences in study variables between obese participants and participants with healthy body weight.

Variable	All participants (n= 140)	Participants with healthy body weight (BMI = 18.5-25 Kg/m^2) (n= 70)	Obese participants (BMI > 30 Kg/m^2) (n= 70)	P-value*
Age (Years)	25.44±4.30	24.91±4.03	25.96±4.52	0.15
BMI (Kg/m^2)	29.22±7.98	22.37±1.90	36.07±5.42	<0.001
Gender				
Male	70 (50)	35 (50)	35 (50)	1.00
Female	70 (50)	35 (50)	35 (50)	
Smoking				
Yes	49 (35)	27 (38.6)	22 (31.4)	0.48
No	91 (65)	43 (61.4)	48 (68.6)	
Marital status				
Single	104 (74.3)	56 (80)	48 (68.6)	0.18
Married	36 (25.7)	14 (20)	22 (31.4)	
Education				
Secondary school	34 (24.3)	17 (24.3)	17 (24.3)	1.00
University	106 (75.7)	53 (75.7)	53 (75.7)	
Employment				
Yes	80 (57.1)	30 (42.9)	30 (42.9)	1.00
No	60 (42.9)	40 (57.1)	40 (57.1)	
Number of family members	6 (4-8)	6 (4.25-8)	6 (4-8)	0.44
Average family income				
≤ 500 JD	65 (46.4)	28 (40)	37 (52.9)	0.19
501 – 1000 JD	62 (44.3)	33 (47.1)	29 (41.4)	
> 1000 JD	13 (9.3)	9 (12.9)	4 (5.7)	
Regular exercise				
Yes	40 (28.6)	17 (24.3)	23 (32.9)	0.35
No	100 (71.4)	53 (75.7)	47 (67.1)	
Family history of CVDs				
Yes	68 (48.6)	31 (44.3)	37 (52.9)	0.40
No	72 (51.4)	39 (55.7)	33 (47.1)	
Family history of DM				
Yes	72 (51.4)	32 (45.7)	40 (57.1)	0.24
No	68 (48.6)	38 (54.3)	30 (42.9)	
SBP (mmHg)	119.06±12.39	114.57±11.23	123.56±11.92	<0.001
SBP (mmHg)				
<120 mmHg	82 (58.6)	54 (77.1)	28 (40)	<0.001
≥120 mmHg	58 (41.4)	16 (22.9)	42 (60)	
DBP (mmHg)	75.91±8.51	72.66±7.78	79.16±7.99	<0.001

Variable	All participants (n= 140)	Participants with healthy body weight (BMI = 18.5-25 Kg/m^2) (n= 70)	Obese participants (BMI > 30 Kg/m^2) (n= 70)	P-value*
DBP (mmHg)				
<80 mmHg	94 (67.1)	59 (84.3)	35 (50)	<0.001
≥80 mmHg	46 (32.9)	11 (15.7)	35 (50)	
Total cholesterol (mg/dL)	169.27±32.35	159.43±26.58	179.11±34.72	<0.001
Total cholesterol (mg/dL)				
<200 mg/dL	120 (85.7)	67 (95.7)	53 (75.7)	0.001
≥200 mg/dL	20 (14.3)	3 (4.3)	17 (23.3)	
HDL (mg/dL)	48.53±11.64	50.96±12.34	46.10±10.42	0.01
HDL (mg/dL)				
≥60 mg/dL (Optimal)	24 (17.1)	17 (24.3)	7 (10)	0.01
40-60 mg/dL (At risk)	79 (56.4)	41 (58.6)	38 (54.3)	
< 40 mg/dL (Dangerous)	37 (26.4)	12 (17.1)	25 (35.7)	
LDL (mg/dL)	99.76±29.85	91.57±31.63	107.94±31.63	<0.01
LDL (mg/dL)				
<130 m/dL (Good)	122 (87.1)	67 (95.7)	55 (78.6)	<0.01
≥130 (borderline-high)	18 (12.9)	3 (4.3)	15 (21.4)	
Triglycerides (mg/dL)	94 (64.50-128.0)	77.50 (58.25-98.50)	111 (85.25-163.75)	<0.001
Triglycerides (mg/dL)				
<150 mg/dL (Optimal)	116 (82.9)	67 (95.7)	49 (70)	<0.001
≥150 mg/dL (Elevated)	24 (17.1)	3 (4.3)	21 (30)	
Glucose (mg/dL)	93.31±17.51	87.77±5.38	98.84±22.94	<0.001
Glucose (mg/dL)				
<100 mg/mL	113 (80.7)	68 (97.1)	45 (64.3)	<0.001
≥100 mg/dL	27 (19.3)	2 (2.9)	25 (35.7)	
Insulin (pg/mL)	629.34 (368.08-1011.43)	390.49 (245.60-656.42)	938.21 (621.85-1433.38)	<0.001
HOMA - IR	1.42 (0.81-2.35)	0.87 (0.54-1.42)	2.26 (1.42-3.23)	<0.001
HOMA – IR				
≤1.9 (Normal)	91 (65)	62 (88.9)	29 (41.4)	<0.001
>1.9 (IR)	49 (35)	8 (11.4)	41 (58.6)	
Homocysteine (pmol/mL)	472.91±180.81	487.47±196.23	458.35±164.08	0.34

* Statistically significant differences between study groups (p-values < 0.05) were determined using Student's t-test or Mann–Whitney U test for continuous variables and Chi-square test for categorical variables. Data are expressed as frequency (%), mean ± standard deviation or median (25th-75th percentiles). BMI; Body Mass Index, JD; Jordanian Dinar, CVDs; Cardiovascular Diseases, DM; Diabetes Mellitus, SBP; Systolic Blood Pressure, DBP; Diastolic Blood Pressure, HDL; High Density Lipoprotein, LDL; Low Density Lipoprotein, HOMA-IR; Homeostatic Model Assessment of Insulin Resistance.

Correlation between cardiovascular and metabolic biomarkers

As shown in Table 2, BMI was significantly correlated with SBP, DBP, fasting glucose, insulin, HOMA-IR, total cholesterol, LDL, TGs, and was significantly inversely correlated with HDL (P-values < 0.01). HOMA-IR was significantly correlated with SBP, DBP, total cholesterol, LDL, and TGs, and was significantly inversely correlated with HDL (P-values < 0.01). SBP was significantly correlated with DBP, insulin, HOMA-IR and TGs, and significantly inversely correlated HDL (p-values < 0.01). DBP was significantly correlated with insulin, HOMA-IR, total cholesterol, LDL and TGs (p-values < 0.01), and significantly inversely correlated with HDL (p-value < 0.05). Fasting glucose was significantly correlated with insulin, HOMA-IR, and TGs (p-values < 0.01) and was significantly inversely correlated with HDL (p-value < 0.05). Insulin was significantly correlated with HOMA-IR, total cholesterol, and TGs (p-values < 0.05). Total cholesterol was significantly correlated with LDL and TGs (p-values < 0.001). HDL was significantly inversely correlated with LDL and TGs p-values < 0.01). LDL was significantly correlated with TGs (p-value < 0.001).

Predictors of cardiovascular risk variables

The predictors of the studied cardiovascular risk variables were identified using multiple linear regression analyses (Table 3). The results showed direct associations between SBP and both BMI and DBP, and an inverse association between SBP and HDL (p-values < 0.05). LDL was directly associated with age and HOMA-IR (p-values < 0.05). HDL was inversely associated with both SBP and TGs (p-values < 0.05). TGs level was directly associated with both BMI and total cholesterol (p-values < 0.05). Total cholesterol was directly associated with age, HOMA-IR, and TGs (p-values < 0.05). Fasting glucose was directly associated with TGs and fasting insulin (P-values < 0.05). Fasting insulin was also directly associated with BMI (p-value < 0.001).

Table 2: Correlations between cardiovascular and metabolic biomarkers

	SBP (mmHg)	**DBP (mmHg)**	**Glucose (mg/dL)**	**insulin (pg/mL)**	**HOMA-IR**	**Total cholesterol (mg/dL)**	**HDL (mg/dL)**	**LDL (mg/dL)**	**Triglycerides (mg/dL)**	**Homocysteine (pmol/mL)**
Age (Years)	0.01	0.19*	0.11	0.02	0.02	0.38***	-0.15	0.36***	0.31***	-0.02
BMI (Kg/m^2)	0.37***	0.36***	0.28***	0.55***	0.58***	0.26**	-0.27**	0.24**	0.52***	-0.04
SBP (mmHg)	-	0.46***	0.06	0.26**	0.27**	0.08	-0.25**	0.13	0.26***	-0.13
DBP (mmHg)	-	-	0.08	0.30***	0.31***	0.23**	-0.20*	0.26**	0.27**	-0.13
Glucose (mg/dL)	-	-	-	0.35***	0.44***	0.09	-0.17*	<0.01	0.25**	0.01
Insulin (pg/mL)	-	-	-	-	0.99***	0.19*	-0.16	0.15	0.41***	0.08
HOMA-IR	-	-	-	-	-	0.25**	-0.23**	0.21**	0.42***	0.05
Total cholesterol	-	-	-	-	-	-	0.02	0.93***	0.46***	-0.08

	SBP (mmHg)	DBP (mmHg)	Glucose (mg/dL)	insulin (pg/mL)	HOMA-IR	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Triglycerides (mg/dL)	Homocysteine (pmol/mL)
(mg/dL)										
HDL (mg/dL)	-	-	-	-	-	-	-	-0.22**	-0.48***	-0.06
LDL (mg/dL)	-	-	-	-	-	-	-	-	0.37***	-0.08
Triglycerides (mg/dL)	-	-	-	-	-	-	-	-	-	0.04

Pearson's or Spearman's correlation test (p-values < 0.05 were considered significant). BMI; Body Mass Index, SBP; Systolic Blood Pressure, DBP; Diastolic Blood Pressure, HDL; High Density Lipoprotein, LDL; Low Density Lipoprotein, HOMA-IR; Homeostatic Model Assessment of Insulin Resistance. * (P-value < 0.05), ** (P-value ≤ 0.01), and *** (P-value < 0.001).

Table 3: Predictors of cardiovascular risk variables

Cardiovascular risk variables (Dependent variables)	R^2	ANOVA	Model	B	β	P-value*
SBP (mmHg)	0.28	F (5,134) = 10.17, p-value < 0.001	Constant	96.64	-	<0.001
			BMI (Healthy body weight vs. obese)	5.50	0.22	0.02
			DBP (mmHg)	0.52	0.36	<0.001
			HOMA-IR (≤1.9 vs. >1.9)	0.16	0.01	0.94
			Log (Triglycerides (mg/dL))	-5.60	-0.09	0.32
			HDL (mg/mL)	-0.19	-0.18	0.04
DBP (mmHg)	0.30	F (7,132) = 8.21, p-value < 0.001	Constant	33.31	-	0.01
			Age (Years)	0.28	0.14	0.09
			BMI (Healthy body weight vs. obese)	2.71	0.16	0.09
			HOMA-IR (≤1.9 vs. >1.9)	1.39	0.08	0.38
			Total cholesterol (mg/dL)	0.02	0.08	0.39
			HDL (mg/mL)	-0.03	-0.04	0.63
			Log (Triglycerides (mg/dL))	0.17	<0.01	0.97
			SBP (mmHg)	0.25	0.37	<0.001
LDL (mg/dL)	0.26	F (6,133) = 7.77, p-value < 0.001	Constant	-21.76	-	0.57
			Age (Years)	2.22	0.32	<0.001

Cardiovascular risk variables (Dependent variables)	**R^2**	**ANOVA**	**Model**	**B**	**β**	**P-value***
			BMI (Healthy body weight vs. obese)	2.09	0.04	0.71
			DBP (mmHg)	0.30	0.08	0.31
			HOMA-IR (≤1.9 vs. >1.9)	13.78	0.22	0.01
			HDL (mg/mL)	-0.19	-0.08	0.38
			Log (Triglycerides (mg/dL))	16.31	0.11	0.26
HDL (mg/dL)	0.26	F (6,133) = 7.86, p-value <0.001	Constant	121.88	-	<0.001
			BMI (Healthy body weight vs. obese)	0.71	0.03	0.75
			SBP (mmHg)	-0.17	-0.18	0.04
			DBP (mmHg)	-0.01	-0.01	0.92
			HOMA-IR (≤1.9 vs. >1.9)	1.41	0.06	0.51
			LDL (mg/dL)	-0.03	-0.07	0.41
			Log (Triglycerides (mg/dL))	-26.14	-0.45	<0.001
Log (Triglycerides (mg/dL))	0.48	F (7,132) = 17.13, p-value <0.001	Constant	1.85	-	<0.001
			Age (Years)	0.01	0.10	0.18
			BMI (Healthy body weight vs. obese)	0.07	0.18	0.02
			SBP (mmHg)	<-0.01	-0.04	0.63
			DBP (mmHg)	<0.01	<0.01	0.97
			HOMA-IR (≤1.9 vs. >1.9)	0.02	0.05	0.54
			Total cholesterol (mg/mL)	<0.01	0.35	<0.001
			HDL (mg/mL)	-0.01	-0.43	<0.001
Total cholesterol (mg/dL)	0.32	F (5,134) = 12.67, p-value <0.001	Constant	-8.89	-	0.78
			Age (Years)	2.31	0.31	<0.001
			BMI (Healthy body weight vs. obese)	2.45	0.04	0.67
			DBP (mmHg)	0.14	0.04	0.63
			HOMA-IR (≤1.9 vs. >1.9)	14.37	0.21	0.01
			Log (Triglycerides (mg/dL))	44.58	0.27	<0.01
Glucose (mg/dL)	0.18	F (4,135) = 7.24, p-value <0.001	Constant	29.90	-	0.17
			BMI (Healthy body weight vs. obese)	4.57	0.13	0.17
			HDL (mg/mL)	0.00	0.00	1.00

Cardiovascular risk variables (Dependent variables)	R^2	ANOVA	Model	B	β	P-value*
			Log (Triglycerides (mg/dL))	18.60	0.21	0.03
			Log (Fasting insulin (pg/mL))	8.81	0.19	0.04
Log (Fasting insulin (pg/mL))	0.32	F (6,133) = 10.45, p-value <0.001	Constant	1.68	-	<0.001
			BMI (Healthy body weight vs. obese)	0.31	0.41	<0.001
			SBP (mmHg)	<0.01	<0.01	0.99
			DBP (mmHg)	<0.01	0.03	0.72
			Glucose (mg/dL)	<0.01	0.16	0.04
			Total cholesterol (mg/mL)	0.00	0.01	0.90
			Log (Triglycerides (mg/dL))	0.24	0.12	0.16

*Multiple linear regression analyses (p-values <0.05 were considered significant). BMI; Body Mass Index, SBP; Systolic Blood Pressure, DBP; Diastolic Blood Pressure, HDL; High Density Lipoprotein, LDL; Low Density Lipoprotein, HOMA-IR; Homeostatic Model Assessment of Insulin Resistance, B; Unstandardized Coefficient, β; Standardized Coefficient, F; F-statistic; R^2; Squared Coefficient of Determination.

Assessment of IR among study participants and association with study variables

The participants were classified into two groups based on their HOMA-IR values. The first group consisted of 91 participants with normal HOMA-IR values (≤ 1.9), while the second group consisted of 49 participants with HOMA-IR values > 1.9 (Early IR). The two groups showed significant differences in several study variables as shown in Table 4.

Table 4: Differences in study variables between participants with HOMA-IR ≤1.9 and participants with HOMA-IR > 1.9

Variable	HOMA – IR ≤ 1.9 (Normal, n= 91)	HOMA – IR > 1.9 (Early insulin resistance, n= 49)	P- value*
Age (Years)	25.75±4.50	24.86±3.88	0.24
BMI (Kg/m^2)	26.18±5.91	34.86±8.29	<0.001
Gender			
Male	45 (49.5)	25 (51)	1.00
Female	46 (50.5)	24 (49)	
Smoking			
Yes	31 (34.1)	18 (36.7)	0.85
No	60 (65.9)	31 (63.3)	
SBP (mmHg)	117.27±11.94	122.39±12.64	0.02
SBP (mmHg)			
<120 mmHg	62 (68.1)	20 (40.8)	<0.01
≥120 mmHg	29 (31.9)	29 (59.2)	
DBP (mmHg)	74.40±8.23	78.71±8.27	<0.01

Variable	HOMA – IR ≤ 1.9 (Normal, n= 91)	HOMA – IR > 1.9 (Early insulin resistance, n= 49)	P- value*
DBP (mmHg) <80 mmHg ≥80 mmHg	 70 (76.9) 21 (23.1)	 24 (49) 25 (51)	 <0.01
Total cholesterol (mg/dL)	162.59±29.13	181.67±34.61	<0.01
Total cholesterol (mg/dL) <200 mg/dL ≥200 mg/dL	 83 (91.2) 8 (8.8)	 37 (75.5) 12 (24.5)	 0.02
HDL (mg/dL)	49.38±12.42	46.94±9.94	0.24
HDL (mg/dL) ≥60 mg/dL (Optimal) 40-60 mg/dL (At risk) < 40 mg/dL (Dangerous)	 19 (20.9) 49 (53.8) 23 (25.3)	 5 (10.2) 30 (61.2) 14 (28.6)	 0.31
LDL (mg/dL)	94.01±25.91	110.43±33.79	<0.01
LDL (mg/dL) <130 m/dL (Good) ≥130 (borderline-high)	 83 (91.2) 8 (8.8)	 39 (79.6) 10 (20.4)	 0.07
Triglycerides (mg/dL)	83 (59-114)	110 (88-137)	<0.01
Triglycerides (mg/dL) <150 mg/dL (Optimal) ≥150 mg/dL (Elevated)	 77 (84.6) 14 (15.4)	 39 (79.6) 10 (20.4)	 0.49
Glucose (mg/dL)	90.70±13.76	98.14±22.27	0.02
Glucose (mg/dL) <100 mg/mL ≥100 mg/dL	 82 (90.1) 9 (9.9)	 31 (63.3) 18 (36.7)	 <0.001
Fasting insulin (pg/mL)	424.35 (271.36-603.35)	1135.24 (968.10-2026.73)	<0.001
Homocysteine (pmol/mL)	469.57±199.05	479.13±142.56	0.77

* Statistically significant differences between participants with HOMA-IR ≤1.9 and participants with HOMA-IR > 1.9 (p-values < 0.05) were determined using Student's t-test or Mann–Whitney U test for continuous variables and Chi-square test for categorical variables. Data are expressed as frequency (%), mean ± standard deviation or median (25^{th}-75^{th} percentiles). BMI; Body Mass Index, SBP; Systolic Blood Pressure, DBP; Diastolic Blood Pressure, HDL; High Density Lipoprotein, LDL; Low Density Lipoprotein, HOMA-IR; Homeostatic Model Assessment of Insulin Resistance.

related to obesity, blood pressure, glucose metabolism, and lipid profile, such as BMI, SBP, DBP, fasting glucose, total cholesterol, LDL, TG, and fasting insulin. The details of these variables are presented in Table 3.4. Participants with early IR had significantly higher levels of BMI, SBP, DBP, total cholesterol, LDL, fasting glucose, and fasting insulin and lower level of HDL compared to participants with normal HOMA-IR (p-values < 0.05).

To find predictors of IR among study participants, further binary logistic regression analysis was performed (Table 5). Results showed that early IR (HOMA-IR> 1.9) can be predicted from obesity (Odds ratio = 8.01, p-value < 0.001).

Table 5: Predictors of HOMA – IR

Variable	Value	B (SE)	Odds ratio	Confidence interval	P-value*
Constant	-	-6.09 (4.15)	-	-	<0.01
SBP (mmHg)	-	<0.01 (0.02)	1.00	0.97-1.04	0.89
DBP (mmHg)	-	0.02 (0.03)	1.02	0.96-1.08	0.57
Total cholesterol (mg/dL)	-	0.01 (0.01)	1.01	1.00-1.03	0.16
Log (Triglycerides (mg/dL))	-	0.32 (1.37)	1.38	0.10-20.15	0.81
HDL (mg/dL)		<0.01 (0.02)	1.00	0.96-1.05	0.91
BMI (Kg/m^2)	Obese Healthy body weight (reference)	2.08 (0.50)	8.01	2.99-21.45	<0.001

*Binary logistic regression (dependent variable: HOMA-IR >1.9 versus HOMA-IR ≤1.9), p<0.05 was considered statistically significant. B: coefficient (intercept); SE: standard error; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high density lipoprotein; BMI, body mass index.

DISCUSSION

This study demonstrated an association between general obesity and increased risk of cardiovascular diseases among young adults. Obese participants had significantly elevated levels of SBP, DBP, total cholesterol, TGs, and LDL, as well as lower levels of HDL. These findings indicate that obese young adults are at higher risk to develop hypertension and dyslipidemia, a leading cause of CVDs. Obese subjects also had significantly higher levels of fasting glucose, fasting insulin, and HOMA-IR. In other words, they had higher levels of IR which, if not treated, may progress to type 2 Diabetes Mellitus. Similar recent study conducted in India found that general obesity among young adults is associated with increased risk of hypertension and dyslipidemia.[24] Another study performed in Kenya to investigate hypertension risk factors among young adults found that obesity and life style factors are the main risk factors.[25] Also, a study conducted over Swedish young women revealed that overweight women showed significantly increased risk for early acute myocardial infarction and ischemic stroke while obese females showed marked increased risk.[26]

One of the main goals of this study was to counter the widespread belief that cardiometabolic disorders can be detected only in older ages. Results of this study proved that these disorders may be detected at early age especially in individuals with higher risk and combined risk factors such as obesity, family history, and sedentary lifestyle. This means that urbanization and western lifestyle with high fat diet full of industrial food along with lack of physical activity and regular exercise as well as elevated levels of stress, altogether may cause an acceleration in cardiometabolic risk development among young adults. Indeed, more research is currently focusing on identifying biological, socioeconomical and environmental factors

contributing to obesity development in young adults. [27, 28]

This knowledge opens the door for stakeholders to come up with preventative actions for fatal cardiometabolic disorders and here are some suggestions; for example, governments should raise the awareness about the importance of overall healthy lifestyles for all people and especially for younger ages. Reliable health and dietary information and statistics should be provided and updated regularly by health authorities and other concerned authorities. Also, governments must impose strict control over the food spread in the markets and its ingredients that may be an underling cause of the development of obesity and related disorders. Accessible health care facilities with dietary consultants should be available for all society segments. As applied by some countries, free places equipped for exercise should be available to encourage people to exercise regularly. Healthcare professionals should update their protocols especially with young obese adults, regular check of SBP, DBP, fasting glucose, HOMA-IR and lipid panel should be conducted at earlier ages, as early detection provides easier, more effective, and less expensive solutions. Finally, the general population should be aware of this risk, especially young adults, they should conduct serious changes to their lifestyle to reduce the elevated risk of cardiometabolic disease development.

This study also confirmed the previous knowledge about the correlation between IR (in terms of HOMA-IR) and other cardiometabolic risk factors such as SBP, DBP, total cholesterol and TGs.[29-31] Participants with IR had a significant higher number of cardiovascular risk factors, in other words, people with IR regardless of their weight status, are at high risk to develop hypertension, dyslipidemia and their correlated cardiometabolic disorders.

One of the remarkable findings of this study is that HDL levels among participants, as 56.4% of them were at risk (40 – 60 mg/dl), and only 17.1% had optimal HDL levels, which indicates an increased risk to develop CVD even among young ages, this decrease in HDL levels may be because of genetic factors, smoking, bad diet, and lack of exercise. However, serious lifestyle changes should be implemented to overcome this risk.

Homocysteine levels were within normal levels for all participants and no significant correlation between homocysteine and cardiovascular risk factors was noted as well. However, conflicting results from research were noticed regarding the correlation between homocysteine and CVDs, as it was suggested to be a marker rather than a cause of CVDs. Several factors may affect hyperhomocysteinemia prevalence among certain population including age, genetics, nutritional status, lifestyle, and environmental factors.[32] A population based cross sectional study performed in China revealed a significant effect of age, BMI, smoking and vegetable consumption on homocysteine levels.[33] Young age of our participants, folic acid fortified food and vegetable consumption may be causes of normal homocysteine levels among all participants.

Together, this study demonstrated the association between obesity among young adults and increased cardiovascular risk and IR compared to subjects with healthy body weight. Therefore, obesity should be considered as a risk factor for cardiometabolic disorders during young adulthood. The study also found an association between IR in terms of (HOMA-IR values) and increased cardiovascular risk including increased SBP, DBP, fasting glucose, total cholesterol, LDL, and TGs, and decreased HDL.

This study has some strengths including its case-control design comparing two groups of obese and healthy weight. Moreover, the selected sample size was adequate to find significant differences in cardiometabolic biomarkers between the study groups. As well, this study and up to the best of our knowledge is the first study that investigated the relationship between obesity and cardiovascular risk biomarkers in Jordan in young healthy adults. Despite these strengths, the study also has some limitations. Overweight individuals were not included because of fund limitations. Collecting information about lifestyle and family history

may affect the certainty of data obtained. Even though, we still believe that the results of this study are valid and further investigations regarding obesity and cardiometabolic risk factors among young adults should be conducted.

CONCLUSIONS

This study showed that obese participants exhibited higher blood pressure, fasting glucose, lipids, and IR compared to healthy body weight participants. IR was correlated with increased blood pressure and lipids. Participants with IR had higher BMI, SBP, DBP, cholesterol, LDL, and TGs compared to participants with normal insulin sensitivity. Increased SBP, TGs, insulin and HOMA-IR were associated with obesity. Therefore, obesity was associated with increased cardiovascular risk and increased prevalence of IR in young adults. Results suggest that obesity should be considered as a predisposing factor to cardiovascular risk and IR. Further studies should be conducted with larger sample size to detect cardiometabolic risk factors among young adults. In addition to observational clinical studies, further studies of genetic factors behind the presented correlations, as well as metabolomic studies should be conducted to find early markers able to detect cardiometabolic risk at younger age.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

MATERIALS AND METHODS

Study design and participants

This was a cross-sectional study that involved apparently healthy 70 obese and 70 matched adults with healthy body weight. The sample was recruited by convenience between Nov 2022 and April 2023 by advertising the study at Jordan University of Science and Technology and King Abdullah University Hospital, Irbid, Jordan. Thus, our sample was recruited from the university students, university employees, hospital employees and visitors of the hospital. Matching between controls and cases was done according to age and sex. A control was chosen and recruited each time a case was recruited. Eligible subjects were approached and informed about the study objectives. Ethical approval was obtained from the International Review Board (IRB) of Jordan University of Science and Technology (JUST, approval No.: 2022/584). The study was conducted in accordance with the World Medical Association Declaration of Helsinki and the ICH Good Clinical Practice guidelines. All subjects who agreed to participate in the study provided written informed consents. Eligibility criteria included apparently healthy young male and female adults aged from 20 to 35 years. For control group, participants' body mass index (BMI) values were between 18.5 and 25.0 Kg/m^2 (healthy weight) and for obese group, BMI values were equal to or more than 30 Kg/m^2. All participants declared that they do not have any acute or chronic illness at the time of participation. Exclusion criteria included pregnant females, patients with malignancies, chronic kidney, heart or liver diseases, patients who received medications for dyslipidemia, diabetes, or hypertension, as well as participants who received medications that affect glucose and insulin levels such as metformin or other hypoglycemic agents.

Sample size calculations

The sample size of cases and controls was calculated using the Power and Sample Size Calculation software version 3.0.34 (Vanderbilt Biostatistics, Vanderbilt University Medical Center, Nashville, USA) based on the previously reported prevalence of obesity among young adults (28.64%) [35] and assuming confidence level of 0.95, odds ratio of 5, expected proportion in controls 0.05, and power of 0.80. Accordingly, a sample size of 67 obese and 67 lean subjects was enough to find significant statistical differences between the two groups. However, we recruited 70 obese and 70 lean subjects to participate in this study.

Data collection and blood sampling

Information about age, gender, smoking, marital status, education, employment, number of family members,

average family income, physical activity, and medical history were collected by self-reporting. Participant weight in kilograms (kg) and height in meters (m) were in order to calculate BMI using the formula (BMI = weight (Kg) / [height (m)]²).[34] Systolic (SBP) and diastolic (DBP) blood pressures were measured using digital sphygmomanometer. After that 10ml of fasting venous blood samples were collected in plain tubes and serum was separated and stored at -20°C for further processing.

Biochemical analyses

All serum samples were tested for insulin and homocysteine using commercially available ELISA kits (Fine Test®, China), tests were performed according to the manufacturer's procedures, optical density (O.D.) absorbance was measured at 450nm using Diatek® microplate reader (Wuxi City, Jiangsu Province, China). All Samples and standards were run in duplicates.

Serum glucose was determined using the commercially available kit from (Bio Research®). Serum Cholesterol, TGs, and HDL were also determined using the commercially available kits from (BioMed®, Hannover, Germany. Absorbance was measured using semi-automated clinical chemistry analyzer (MISPAVIVA® by AGAPPE, Switzerland). All Samples were run in duplicates.

LDL levels were determined using the Friedewald equation [LDL Cholesterol (mg/dl) = Total Cholesterol-(Triglycerides/5) - HDL Cholesterol].[35]

Insulin resistance homeostasis model assessment (HOMA-IR) was calculated from fasting serum insulin and glucose levels using the formula [HOMA-IR = fasting insulin (mIU/mL)*fasting glucose (mg/dl)/405] to estimate IR.[36] Participants with HOMA-IR levels more than 1.9 were considered as having early IR.[37]

Statistical analyses

Statistical analyses were conducted using the IBM SPSS statistics software version 25 (Armonk,NY, USA). Statistical significance was determined at $p < 0.05$. Normality was tested first by the Shapiro-Wilk test, eye inspection of the Q-Q plot, and histogram with normal curves. Continuous variables were reported as average± standard deviation (SD) or median (25^{th}-75^{th} percentiles) as appropriate. Categorical variables were presented with frequency and percentage. The relationship between continuous variables were examined using the Pearson's or Spearman's correlation test as appropriate. Differences in categorical and continuous variables between obese participants and participants with healthy body weight were determined using Student's t-test, Mann-Whitney U-test, or Chi square test as appropriate. Differences in categorical and continuous variables between participants with HOMA-IR ≤ 1.9 and participants with HOMA-IR > 1.9 were determined using Student's t-test, Mann-Whitney U-test, or Chi square test as appropriate. Multiple linear regression analyses were used to identify predictors of cardiovascular risk parameters. Binary logistic regression analysis was used to identify predictors of HOMA-IR.

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Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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ارتباط السمنة بزيادة خطر الإصابة بأمراض القلب وارتفاع معدل مقاومة الإنسولين بين البالغين الشباب الأصحاء ظاهريًا

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ملخص

الهدف: دراسة العلاقة بين السمنة وعوامل خطر الأمراض القلبية والأيضية ومقاومة الأنسلين لدى البالغين الشباب الأصحاء.

الطرق: شملت هذه الدراسة المقطعية 70 بالغًا بدينًا و70 بالغًا متناسبين للعمر والجنس بوزن جسم طبيعي. تم قياس الجلوكوز في المصل، والأنسولين، والدهون، والهوموسيستئين. تم تحديد مقاومة الأنسلين باستخدام تقييم نموذج الاستقرار الذاتي.(HOMA-IR) IR- كم تم قياس ضغط الدم الانقباضي والانبساطي .

النتائج: أظهر المشاركون البالغون البدناء زيادة في قراءات ضغط الدم ومستويات الجلوكوز والتريغليسيريدات والكوليسترول واللیبوبروتين ذي الكثافة المنخفضة (LDL) والأنسولين وHOMA-IR ، وانخفاض في الليبوبروتين ذي الكثافة العالية (HDL) مقارنة بالمشاركين ذوي الوزن الطبيعي. كما كان مؤشر كتلة الجسم (BMI) مرتبطًا طردياً بـ قراءات ضغط الدم والجلوكوز والأنسولين و HOMA-IR والكوليسترول والتريغليسريدات، وكان ارتباطاً عكسياً بالكولستيرول ذو الكثافة العالية. إضافة لذلك، كان HOMA-IR مرتبطًا طردياً بقراءات الضغط والتريغليسيريدات والكولستيرول ذو الكثافة المنخفضة، وارتباطاً عكسياً بالكوليسترول ذو الكثافة العالية. كان لدى المشاركين الذين يعانون من مقاومة الانسلين قيم أعلى لمؤشر كتلة الجسم وضغط الدم والتريغليسيريدات والكولستيرول ذو الكثافة المنخفضة مقارنة بالمشاركين ذوي الحساسية الطبيعية للأنسولين. كانت السمنة مرتبطة بزيادة قراءات ضغط الدم الإنقباضي والتريغليسريدات ومستويات الأنسولين ومقاومة الأنسولين. لم يكن هناك فرق كبير في الهوموسيستئين بين المجموعات.

الاستنتاج: السمنة مرتبطة بزيادة خطر الأمراض القلبية وزيادة انتشارمقاومة الانسولين بين البالغين الشباب الأصحاء. يتطلب ذلك القيام بالتدخلات الدوائية والسلوكية بشكل عاجل لمعالجة زيادة مخاطر الأمراض القلبية في هذه الفئة العمرية.

الكلمات الدالة: السمنة؛ البالغون الشباب؛ خطر الإصابة بأمراض القلب؛ مقاومة الإنسولين.

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Phytochemical Screening and Evaluation of Ameliorating Effect of *Aleuritopteris bicolor* (Roxb.) Fraser-Jenk Leaves Extract on Renal and Hepatic Impairment in a Rat Model of Gentamicin-Induced Renal Toxicity

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ABSTRACT

Background: The fern species *Aleuritopteris bicolor* (AB), in the Pteridaceae family, traditionally used for wound healing and treating various ailments.

Aim: This study was conducted to evaluate *Aleuritopteris bicolor* (AB) hydro ethanol leaf extract potential in mitigating gentamicin-induced nephrotoxicity and hepatotoxicity in Albino rats.

Methods and Materials: This study was achieved by performing a phytochemical test on AB hydro ethanol leaves extract and administering hydro ethanol extract of AB leaves orally and gentamicin (80 mg/kg/day) intraperitoneal for a period of seven consecutive days to male albino rats, followed by analysis of biochemical function, histopathology, and the weight of the kidney and liver after the eighth day. SPSS version 23 was used for data analysis. Results were presented as mean ± standard deviation (n=6). Statistical analysis involved one-way ANOVA followed by post hoc least significant difference (LSD) test.

Results: From the phytochemical screening, *Aleuritopteris bicolor (*AB) hydro ethanol extract was found to contain flavonoids, phenols, saponins, and tannins. Acute toxicity testing showed its safety up to 5000 mg/kg. Gentamicin administration (Group II) resulted in a significant ($p < 0.001$) increase in urea (154.07 ± 6.22 mg/dl), creatinine (8.90 ± 0.51 mg/dl), and uric acid (3.27 ± 0.74 mg/dl), indicating renal dysfunction compared to the negative control group (distilled water). Co-treatment with ascorbic acid (Group III) and varying doses of *Aleuritopteris bicolor (*AB) extract (Groups IV, V, and VI) led to significant reductions in urea and creatinine levels, with the 500 mg/kg AB extract dose showing the most notable effects ($p < 0.001$) compared to the gentamicin-only group. Histopathological analysis revealed that gentamicin caused tubular degeneration, colloid cast formation, and glomerular injury, while treatment with AB extract minimized these damages. Additionally, gentamicin caused significant increases in serum ALP ($p < 0.001$), AST ($p < 0.001$), and ALT ($p < 0.001$) compared to the control group. Treatment with AB extract significantly reduced these enzymes ($p < 0.001$) compared to the gentamicin-only group. Histological analysis showed the gentamicin group had portal inflammation and hepatocyte degeneration, while AB extract minimized these changes, supporting its protective effects against renal and hepatic toxicity.

Conclusions: These findings suggest that *Aleuritopteris bicolor* extract effectively mitigates gentamicin –induced nephrotoxicity and hepatotoxicity in Albino rats, demonstrating potential for therapeutic use.

Keywords: *Aleuritopteris bicolor*, phytochemical analysis, renal protective effects, hepatoprotective effects, gentamicin toxicity.

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INTRODUCTION

Nephrotoxicity refers to kidney damage caused by drugs and chemicals and is a significant contributor to chronic kidney disease, affecting more than 27 million individuals (1). It often arises as a result of acute kidney injury, with chemical-induced nephrotoxicity accounting for approximately 20% of these cases (2). Prolonged use of the antibiotic such as gentamicin can lead to kidney damage by generating oxidative stress within the kidney cells. This damage impairs the functioning of proteins, lipids, and DNA, triggering inflammation and disrupting the transport of sodium, which ultimately causes cellular swelling and death (3). Similarly, gentamicin alters various body chemicals such as liver enzymes, creatinine, electrolytes, uric acid, urea, protein, and inflammatory substances. Kidney injuries can also impact other organs, including the liver. Renal ischemia-reperfusion injury can occur, leading to liver damage characterized by leukocyte infiltration, congestion, and cellular necrosis. Hepatotoxicity, caused by drugs, is a major global healthcare concern, responsible for 75% of fatal adverse drug reactions and 5% of hospitalizations.(4)

Certain fern species such as *Adiantum capillus, Dryopteris filix-mas, Polypodium lecotomos* are documented to possess hepatoprotective while *Adiantum capillus* is also recognized for its nephroprotective effects against the toxic impacts of chemicals (6,7). Among various fern species, *Aleuritopteris bicolor* (AB) (Roxb.) Fraser-Jenk is traditionally used for improving kidney function in animals as well as treating gastrointestinal diseases in human within the local community of Lekhnath Pokhara and by specific ethnic groups in Nepal(8). However, there exists a scientific gap in understanding the therapeutic potency of *Aleuritopteris bicolor* (AB) for kidney function. To address this gap, further research is essential to explore the potential therapeutic benefit of *Aleuritopteris bicolor* (AB) on kidney and liver functions.

METHODS AND MATERIAL:

Plant Materials Collection

The leaves of *Aleuritopteris bicolor* AB hydroethanol extract were dried in the shade after being collected from a field in Kaski, Lekhnath, Pokhara, Nepal. It was identified by Mr. Dhan Raj Kandel, Scientific Officer, the National Herbarium and Plant Laboratory, Godavari, Lalitpur, Nepal. A voucher specimen (no. PUH-2022-39) was stored in the herbarium section of School of Health and Allied Sciences, Pokhara University, Nepal. Samples of plant leaves were ground up and used for extraction.

Preparation of Extract of Plant Species

Using the method of cold maceration with 70% ethanol in a ratio of 1: 10 (powdered crude drug: solvent = 100 gm : 1000 ml), leaf powder was macerated for three days in a row in a dark area. The resulting extract was then filtered through Whatman filter paper. The obtained filtrates were concentrated in a rotary evaporator at 40°C, and the extracted product was stored in a vacuum desiccator until it was needed.(9)

Phytochemical Screening

According to established procedures, the obtained crude extract underwent a phytochemical analysis to identify the presence of alkaloids, flavonoids, phenols, terpenoids, tannins, glycosides, and reducing sugar. Any appearance of color, change in color, or precipitate formation was used as a sign that these tests had been successful(10).

Studies animals

Fifty-four male albino rats (200–270 g) were obtained from the animal house department of plant resources, Kathmandu, Nepal. They were housed in normal cages at room temperature (25 ± 3 °C, 55 ± 5% humidity, 12 h natural light-dark cycle) at the primate facility of the University of Pokhara, Nepal to acclimate with standard pellets and water and were housed for a week. All tests and procedures were performed in accordance with Organization for Economic Co-operation and Development (OECD) guidelines and approved by the Ethics Committee of the Institutional Review Committee (IRC), Pokhara University Research Center with reference number (02/079/080).

Acute toxicity studies

An acute oral toxicity study, structured according to the fixed dose procedure following OECD 420 guideline, aimed to systematically assess the acute toxicity of the AB extract in male albino rats(11). The study protocol included an overnight fasting period with water access for the rats. On the following day, three groups each consisting of five rats, received a single oral dose of the AB extract at 1000 mg/kg, 3000 mg/kg, and 5000 mg/kg in the morning. Following, individual rats were closely observed at 60, 120, and 240 minutes after administration to monitor immediate effects or signs of toxicity. Surviving rats were continuously monitored for 14 days to detect delayed signs or severity of observed toxicity and mortality(11) .

In vivo experimental design

The improvement in liver and kidney function during acute renal toxicity by the test samples was investigated using a method previously described for gentamicin (GM)-induced nephrotoxicity .(12) Thirty six male albino rats (200–270 g) were weighed, divided into six groups (n = 6), and was acclimatized with sufficient fresh water and food for one week.

The animals included in the study were randomly assigned to six equal groups (n=6) as follows:

Group I: Control (distilled water, 1 ml /kg/day orally) for 7 days. Rats in this group received distilled water (1 ml/kg/day) orally for 7 days. This served as the baseline group with no exposure to gentamicin or test substances.

Group II: Gentamicin (80 mg/kg/day I.P) for 7 days. This group was designed to induce nephrotoxicity and serve as the toxic control.

Group III: Ascorbic acid (200 mg/kg/day orally) + Gentamicin (80mg/kg/day I.P.) for 7 days. This group aimed to test the protective effect of ascorbic acid against gentamicin-induced nephrotoxicity.

Group IV: Extract (125 mg/kg/day orally) + gentamicin (80mg/kg/day I.P.) for 7 days. This group aimed to assess the protective effects of the lower concentration of the extract.

Group V: Extract (250 mg/kg/day orally) + Gentamicin (80mg/kg/day I.P.) for 7 days. This group aimed to assess the protective effects of the moderate concentration of the extract.

Group VI: Extract (500 mg/kg/day orally) + Gentamicin (80mg/kg/day I.P.) for 7 days.This group aimed to assess the protective effects of the higher concentration of the extract.

Biochemical Estimation:

During the 7-day experimental period, daily administrations were conducted. Blood samples were collected with the help of heparin-coated capillaries from the retro-orbital sinus on day 8 under mild anesthesia using ether. The collected blood was allowed to coagulate, and serum was separated by centrifugation at 3000 rpm for 10 minutes. Serum samples were then analyzed for kidney function markers including urea, creatinine, and uric acid, as well as liver enzymes (ALT, AST, and ALP) to assess the impact of gentamicin toxicity and the protective effect of the test substances. A diagnostic kit from thermo fisher scientific and sigma-aldrich was used for each assessment in accordance with the manufacturer's instructions (12).

Histopathological Examination:

Kidney and liver weights were measured at the end of the study by excising the organs post-mortem and expressing them as absolute weights. Both kidneys and liver were gathered, weighed, and preserved for at least 24 hours in 10% buffered formaldehyde. Similarly liver and kidney samples were dehydrated with ethanol (80%), cleared in xylene and was embedded in paraffin wax. Microtome was used to carve a section of tissue measuring 5 mm. Hematoxylin and eosin were used to stain the sections in order to detect histopathological alterations under a 10X microscope to evaluate morphological changes. (12) The results from all experimental groups were compared to the control and gentamicin-only groups to determine the level of protection offered by ascorbic acid and the plant extract against gentamicin-induced toxicity.

Statistical analysis

IBM Statistical Package for Social Sciences (SPSS) software version 23 was used for data analysis, and all data were expressed as mean ± standard deviation (n=6), and statistical analysis was conducted using one-way ANOVA followed by a post hoc LSD (Least significant difference) test. The significant effects are highlighted based on the provided p-values

RESULTS AND DISCUSSIONPHYTOCHEMICAL TEST

The extracts from the plant species *Aleuritopteris bicolor*(AB) was subjected to various phytochemical tests and the tests revelaed the presence of flavonoids, phenols, saponins, and tannins as indicated in **table 1**

Table 1. Qualitative phytochemical data of plant species extract

Phytochemical Constituent	Results of AB extract
Alkaloids	-
Flavonoids	+
Phenols	+
Glycosides	-
Saponins	+
Carbohydrates	-

Note: + present, - absent

The plant *Aleuritopteris bicolor* (AB) has been used as a traditional medicinal for treating different ailment such as wound healing, respiratory diseases, rheumatic conditions and digestive disorder.(13) In the present investigation, phytochemical screening of AB extract revealed the presence of flavonoids, phenols, saponins, and tannins, while alkaloids, glycosides, and carbohydrates were not detected in table 1. Flavonoids and phenols are known for their antioxidant properties and have been associated with various health benefits. They possess potential anti-inflammatory, anti-cancer, and neuroprotective, nephroprotective, hepatoprotective effects(14) · Studies on the hepatoprotective effects of flavonoids have shown their ability to mitigate liver damage and support liver function(15, 16). Furthermore saponins and tannins have been reported to possess anti-inflammatory, immunomodulatory and wound healing activities respectively(17) (18). This suggest that the presence of flavonoids, phenols, saponins and tannins in the AB extract might contribute to its tissue protective, alleviate inflammatory conditions, regulate immune response and promote tissue repair through collagen formation. The presence of flavonoids and phenols in the AB extract suggests that it may have potential therapeutic properties.

Acute toxicity studies

No toxic effects or behavioral changes were observed in rats at fixed dose of 1000 mg/kg, 3000 mg/kg, and 5000 mg/kg during monitoring periods of 60, 120, and 240 minutes, as well as up to 14 days. Additionally, no mortality was observed at the highest administered dose of 5000mg/kg. This absence of mortality suggests that the LD_{50} (lethal dose for 50% of the test animals) would exhibit toxicity or mortality, exceeds the highest tested dose of the extract 5000 mg/kg).In terms of acute toxicity, the absence of toxic effects and behavioural changes in rats at fixed doses of 1000 mg/kg, 3000mg/kg and 5000mg/kg, observed throughout the monitoring periods of periods of 60, 120, and 240 minutes, as well as up to 14 days, indicates a notable safety profile for the *Aleuritopteris*

bicolor (AB) extract in acute oral exposure. The consistent lack of adverse reactions suggests that the extract even at the highest tested dose of 5000mg/kg, doesn't elicit harmful physiological or behavioural responses in male albino rats. Furthermore, the absence of mortality at the highest administered dose reinforces the conclusion that the LD_{50}, representing the lethal dose for 50% of the test animals, surpasses the highest tested dose of the extract. This outcome indicates an absence of acute toxicity of the AB extract up to 5000mg/kg, when administered orally. Previous study conducted in acute toxicity of *Polypodium feei* root extract revealed no adverse effects in rats up to 5000mg/kg, supporting the present data of a low toxicity potential for fern species (19).

Effect of AB extract on serum creatinine, urea, and uric acid

As shown in the Table 2, administration of gentamicin (Group II) led to a significant $p< 0.001$, increase in urea, and creatinine levels, indicating renal dysfunction. However, treatment with ascorbic acid in combination with gentamicin (Group III) resulted in increase in these biomarkers compared to the GM group. The groups treated with various doses of the extract alongside gentamicin (Groups IV, V, and VI) showed significant ($p< 0.001$), reductions in urea, and creatinine levels compared to the GM group, indicating potential properties of the extract. Notably, the highest dose of the extract (500mg/kg) demonstrated the most significant $p< 0.001$, decrease in creatinine levels. Similarly, gentamicin (Group II) led to a significant elevation in uric acid levels compared to the negative control (Group I), while co-treatment with ascorbic acid and various dose of plant extract didn't show any significant control in elevated uric acid level.

Table 2. Data representing level of serum creatinine, uric acid and urea in different groups of treatment

Experimental Group		Serum concentration (mg/dl) of		
		Uric acid	**Urea**	**Creatinine**
Group I	Negative Control (Distilled water)	$2.86\pm0.73^{\#\#\#}$	$46.36\pm2.3^{\#\#\#}$	$1.75\pm0.40^{\#\#\#}$
Group II	Gentamicin	3.27 ± 0.74	$154.07\pm 6.22^{***}$	$8.90 \pm 0.51^{***}$
Group III	Ascorbic acid + Gentamicin	3.56 ± 0.84	$128.99 \pm4.42^{***\#\#\#}$	$6.82 \pm 0.47^{***\#\#\#}$
Group IV	125mg/kg extract + Gentamicin	3.01 ± 0.67	$134.00 \pm 7.18^{***\#\#\#}$	$6.84 \pm 0.10^{***\#\#\#}$
Group V	250mg/kg extract + Gentamicin	3.23 ± 0.72	$117.04 \pm3.82^{***\#\#\#}$	$6.82 \pm 0.49^{***\#\#\#}$
Group VI	500mg/kg extract + Gentamicin	2.83 ± 0.66	$89.27 \pm 7.27^{***\#\#\#}$	$3.49\pm0.26^{***\#\#\#}$

Note: Data expressed as mean ± standard deviation (n=6). One –way ANOVA followed by a post hoc LSD test was used for comparison between different groups. * Significant change in comparison with the control group at $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, # significant change in comparison with the GM group at $\#p< 0.05$, $^{\#\#}p< 0.01$, $^{\#\ \#\#}p< 0.001$.

Gentamicin is an antibiotic commonly used to treat various bacterial infections. The adverse effects associated with gentamicin use is nephrotoxicity, as refers to kidney damage,(20) which involves renal accumulation of the drug, generation of reactive oxygen species, mitochondrial dysfunction, apoptosis, inflammation and increased in creatinine, urea, uric acid etc.(21) Gentamicin can disrupt the normal fluid balance within the kidney, leading to the accumulation of interstitial fluid. This fluid build-up can contribute to the increase in kidney weight.(22)In present study, effect of treatments on biochemical parameter, weight, histostructure of kidney and liver was assessed. Based on the data presented in Table 2, it can be concluded that administration of gentamicin (Group II) resulted in

renal dysfunction, as evidenced by significant increase in urea and creatinine levels. This aligns with previous finding that have consistently reported the nephrotoxic effects of gentamicin (23). However, treatment with ascorbic acid in combination with gentamicin (Group III) and various doses of the extract alongside gentamicin (Groups IV, V, and VI) showed renoprotective properties. In terms of urea and creatinine levels, Group III (ascorbic acid + gentamicin) exhibited increased compared to Group II (gentamicin only). This suggests that ascorbic acid may have a protective effect against gentamicin-induced renal dysfunction. This finding is consistent with studies that have explored the antioxidant properties of ascorbic acid and its ability to counteract oxidative stress- induced renal damage (24). Furthermore, Groups IV, V, and VI, which received different doses of the extract along with gentamicin, demonstrated significant reductions in urea and creatinine levels compared to Group II. These findings indicate the potential renoprotective properties of the extract, with the highest dose (500mg/kg) showing the most significant decreased in creatinine levels, Table 2. Comparing these findings with other research studies, our results aligns with results of other plant species like *Adiantum capillus* a fern extract demonstrated the renoprotective effects against cisplatin-induced nephropathy(7). Similarly, the antioxidant and anti-inflammatory properties of various phytoconstituents have been documented in studies supporting the studies, that natural compounds may offer protection against drug-induced nephrotoxicity (25) (26) (27).

Effect of AB extract on kidney weight

The data presented in Table 3, represents the percentage weight of rat kidneys (right and left) in different treatment groups. As the results indicate that treatment with Gentamicin (Group II) led to a significant increase in the weight of both the right and left kidneys compared to the negative control group (Group I). Group III received Ascorbic acid along with Gentamicin, but no significant difference in kidney weight was observed compared to the Gentamicin group. Groups IV, V, and VI received different doses of an extract in combination with Gentamicin. Group IV (125mg/kg extract + Gentamicin) and) did not show a significant difference in kidney weight compared to the Gentamicin group. However, Group V (250mg/kg extract + Gentamicin) and Group VI (500mg/kg extract + Gentamicin) demonstrated a significant $p< 0.05$, $p< 0.01$ decreased in the weight of both kidneys respectively, compared to the Gentamicin group.

Table 3. Data representing the % weight of rat kidney (right and left) in different group of treatment

Experimental Group		Weight of Kidney (% Absolute value)	
		Right Kidney	**Left Kidney**
Group I	Negative Control (Distilled water)	$0.33\pm0.055^{\#\#}$	$0.31\pm0.04^{\#\#}$
Group II	Gentamicin	$0.55\pm0.00^{**}$	$0.53\pm0.01^{**}$
Group III	Ascorbic acid + Gentamicin	$0.54\pm0.017^{**}$	$0.52\pm0.005^{**}$
Group IV	125mg/kg extract + Gentamicin	$0.52\pm0.005^{**}$	$0.5\pm0.01^{**}$
Group V	250mg/kg extract + Gentamicin	$0.5\pm0.02^{**\#}$	$0.48\pm0.001^{**\#}$
Group VI	500mg/kg extract + Gentamicin	$0.41\pm0.05^{**\#\#}$	$0.4\pm0.005^{**\#\#}$

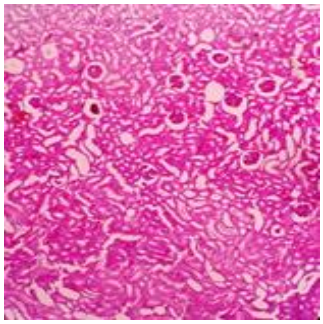
Note: Data expressed as mean ± standard deviation (n=6). One –way ANOVA followed by a post hoc LSD test was used for comparison between different groups. * Significant change in comparison with the control group at $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, # significant change in comparison with the GM group at $\#p< 0.05$, $^{\#\#}p< 0.01$, $^{\# \#}p< 0.01$.

The percentage weight of the kidneys in different treatment groups also provides insights into the renal effects as shown in Table 3. Group II (gentamicin) showed a significant increase in the weight of both the right and left kidneys compared to the negative control group (Group I). This increased in kidney weight suggests kidney enlargement and possibly renal damage. This finding is consistent with previous studies that have reported an association between inncreased kidney weight and nephrotoxic effects of gentamicin (28) (29). Group III (ascorbic acid + gentamicin) did not show a significant difference in kidney weight compared to Group II, indicating that ascorbic acid may not have a significant impact on kidney size in the presence of gentamicin-induced damage. This contrasts with the study on ascorbic acid which reported a reduction in kidney weight following ascorbic acid administration in a model of renal injury (30). However, Groups V and VI, which received higher doses of the extract (250mg/kg and 500mg/kg, respectively) along with gentamicin, demonstrated significant decreases in the weight of both kidneys compared to Group II Table 3. This suggests that the extract may have a dose-dependent effect on reducing kidney size and potentially protecting against gentamicin-induced kidney enlargement. These finding align with studies, which demonstrated the ability of plant extracts to attenuate kidney hypertrophy in experimental model (31).

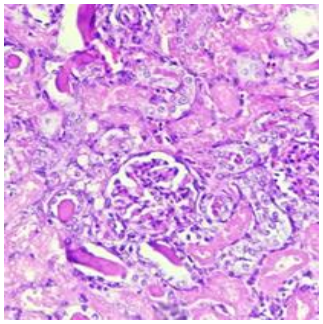
Effect of AB extract on kidney tissue

Histopathological examination of kidney sections

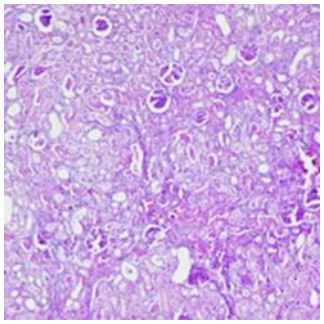
As shown in **Figure 1,** Group II (Gentamicin) shows degenerated tubules, colloid cast with chronic inflammatory cells in the interstitium, and acute glomerular injury. These findings indicate severe kidney damage and inflammation. Compare to this group, Group I and Group VI shows normal kidney parameters without any abnormalities. Group V shows mild glomerular injury, tubular cast, and mild chronic inflammatory cells in the interstitium, which was less severe than the findings in Group III. Additionally, in Group IV, tubular degeneration and chronic inflammatory cells was observed in the interstitium which was similar to Group III. However, there is no presence of glomerular injury in Group IV, distinguishing it from Group III, whereas Group Shows, focal fibrosis and mild chronic inflammatory cells in the interstitium. Compared to Group III, Group V shows some scarring and inflammation in the kidney, but without the acute glomerular injury seen in Group III. Group III displays chronic inflammation in the interstitium, along with degenerated tubules and colloid cast formation. The presence of ongoing inflammation and degenerative changes in the kidney suggests a similar severity of damage as seen in the Gentamicin group (Group II).



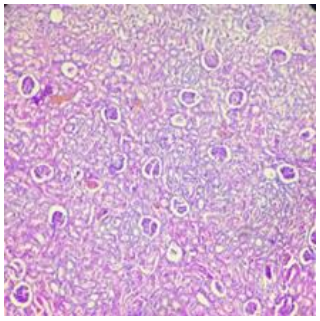
Negative Control(Distilled water)



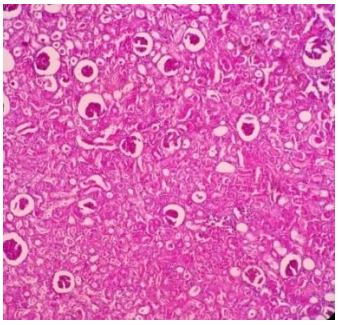
Genta(80mg/kg)



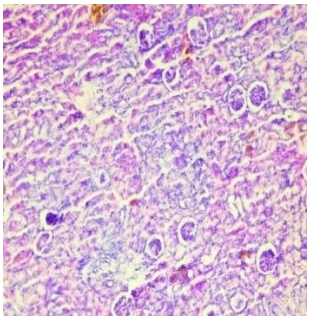
Ascorbic acid (200 mg/kg) and Genta (80 mg/kg)



500mg/kg Extract and Genta (80mg/kg)



250 mg/kg Extract and Genta (80mg/kg)



125 mg/kg Extract and Genta (80mg/kg)

Figure 1. Effect of on kidney histomorphology in various groups

In **Figure 1**, the histopathological analysis of the kidneys further supports the findings. Group III (gentamicin) exhibited severe kidney damage and inflammation, including degenerated tubules, colloid cast formation with chronic inflammatory cells in the interstitium, and acute glomerular injury, align with the well- documented nephrotoxic effects of gentamicin reported in previous studies (28).In contrast, Groups I, which represent the negative control groups, showed normal kidney parameters without any abnormalities. Group IV showed similar tubular degeneration and chronic inflammatory cell presence in the interstitium compared to Group III, but without glomerular injury. This suggests that the extract may have a protective effect on glomerular function. A finding consistent with the previous work which reported the nephroprotective effects of various plant extracts against glomerular injury (31). Group V, demonstrated mild glomerular injury, tubular cast formation, and mild chronic inflammatory cells in the interstitium, indicating some kidney damage and inflammation, although less severe than in Group III. Group VI showed focal fibrosis and mild chronic inflammatory cells in the interstitium, suggesting scarring and inflammation, but without acute glomerular injury. Similar observations were made by other study investigating the protective effects of natural compounds against chemical induced kidney tissue damage (31). As reported in previous study, some of the fern species like *Drynaria quercifolia* , consists of naringin , a flavonoid compound, which has been reported to protect against kidney injury by reducing oxidative stress, inflammation, and apoptosis (programmed cell death) in the kidneys (32).

Effect of AB extract on serum ALP, AST, and ALT

The Table 4, represents the serum concentrations of three enzymes, ALP, AST and ALT in different treatment groups. Group II, which received treatment with Gentamicin, exhibited a significant ($p<0.001$), increased in ALP, AST, and ALT levels compared to the control group. This suggests that Gentamicin administration had a detrimental effect on liver function, as indicated by the elevated levels of these enzymes. In Group IV, V, and VI, which received different doses of the tested extract along with Gentamicin, significant improvements ($p<0.001$), in ALP, AST levels were observed compared to the Group II. Moreover, Group III which received a combination of Ascorbic acid and Gentamicin, showed higher significant decreased ($p<0.001$) in ALP, AST levels when compared to the Gentamicin-only group. These findings suggested that the addition of Ascorbic acid to Gentamicin treatment had a protective effect on the liver.

Table 4. Data representing level of serum ALP, AST, ALT in different groups of treatment

Experimental Group		Serum concentration (mg/dl) of		
		ALP	**AST**	**ALT**
Group I	Negative Control (Distilled water)	$433.86 \pm 14.23^{\#\#\#}$	$62.06 \pm 4.51^{\#\#\#}$	$32.15 \pm 12.21^{\#}$
Group II	Gentamicin	$889.82 \pm 12.348^{***}$	$151.08 \pm 8.898^{***}$	$102.59 \pm 6.46^{***}$
Group III	Ascorbic acid + Gentamicin	$378.61 \pm 13.108^{***\#\#\#}$	$118.10 \pm 9.76^{***\#\#\#}$	75.54 ± 6.59
Group IV	125mg/kg extract + Gentamicin	$750 \pm 11.07^{***\#\#\#}$	$139.69 \pm 5.12^{***\#}$	73.59 ± 19.54
Group V	250mg/kg extract + Gentamicin	$717.79 \pm 12.19^{***\#\#\#}$	$122.30 \pm 5.41^{***\#\#\#}$	86.93 ± 5.6
Group VI	500mg/kg extract + Gentamicin	$685.99 \pm 5.23^{***\#\#\#}$	$129.37 \pm 7.32^{***\#\#\#}$	76.98 ± 4.36

Note: Data expressed as mean ± standard deviation (n=6). One –way ANOVA followed by a post hoc LSD test was used for comparison between different groups. * Significant change in comparison with the control group at $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, # significant change in comparison with the GM group at $\#p< 0.05$, $^{\#\#}p< 0.01$, $^{\#\#}p< 0.001$

In certain cases of prolonged nephrotoxicity, there can be secondary effects on the liver due to the systemic effects of renal dysfunction. If the kidneys are unable to adequately excrete waste products and toxins, it can put additional stress on the liver, leading to alterations in liver biochemical parameters. These changes may include elevated liver enzymes such as ALT, AST, , ALP and bilirubin levels.(33) In present study, similar results were observed in Table 4, after the administration of Gentamicin had a detrimental effect on liver function, as indicated by the significant increase in the levels of three enzymes (ALP, AST, and ALT) in Group II compared to the control group. This implies that Gentamicin treatment alone caused indirect effect in liver through due to nephrotoxicity. This indirect effect on the liver due to gentamicin –induced nephrotoxicity aligns with previous studies emphasizing the interconnectedness of kidney and liver function(34). However, the subsequent treatment groups showed improvements in liver function compared to Group II. In Group IV, V, and VI, which received different doses of the tested extract along with Gentamicin, significant improvements in ALP and AST levels were observed. This suggests that the tested extract had a protective effect on the liver and helped mitigate the adverse effects caused by Gentamicin induced nephrotoxicity. Additionally, Group III, which received a combination of Ascorbic acid and Gentamicin, showed significant decreases in ALP and AST levels compared to the Gentamicin-only group, Table 4. This finding suggests that the addition of Ascorbic acid to Gentamicin treatment had a further protective effect on liver function. This supports the ideas that ascorbic acid may have a protective effect on liver function, consistent with studies that have highlighted its antioxidant properties and ability to mitigate oxidative stress –induced liver damage (35).

Effect of AB extract on liver weight

As shown in Table 5, there observed that, the treatments employed in this study did not have a significant impact on the percentage change in liver weight relative to body weight.

Table 5. Data representing the % weight of rat liver in different group of treatment

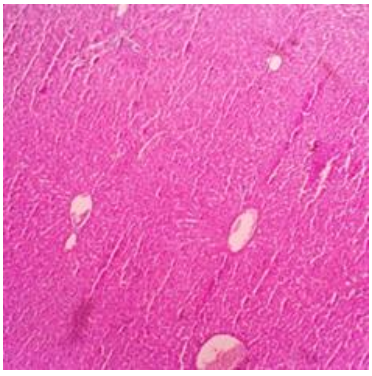
Experimental Group		Weight of liver of body weight (%) liver weight
Group I	Negative Control (Distilled water)	3.61±0.55
Group II	Gentamicin	3.59±0.72
Group III	Ascorbic acid + Gentamicin	4.01±0.06
Group IV	125 mg/kg extract + Gentamicin	4.03±0.69
Group V	250 mg/kg extract + Gentamicin	3.60±0.04
Group VI	500 mg/kg extract + Gentamicin	3.50±0.13

The Table 5 showed, that the treatments did not have a significant impact on the percentage change in liver weight relative to body weight, indicating that the treatments did not cause significant alterations in liver size.

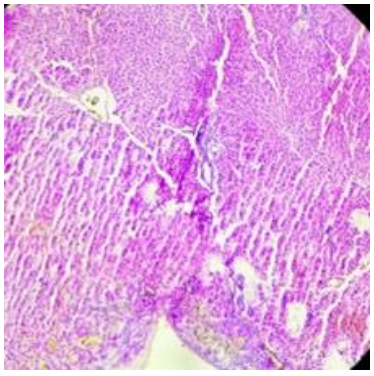
Effect of AB extract on Liver tissue

Histopathological examination of liver sections

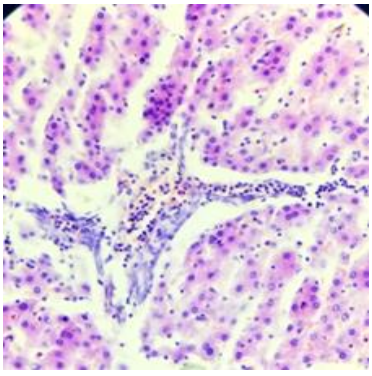
As shown in Figure **2,** Negative control (Group I) showed a normal liver architecture with no abnormalities, indicating the absence of any adverse effects. The Group II (Gentamicin only) demonstrated portal chronic inflammation, bile stasis, and degenerated hepatocytes. The group VI (500mg/kg extract + Gentamicin) displayed mild chronic inflammatory cells and a congested central vein. Similarly, Group III (Ascorbic acid + Gentamicin) had a normal liver architecture with mild chronic inflammation and congested blood vessels. Whereas, Group V (250mg/kg extract + Gentamicin) exhibited mild chronic inflammation in the periportal area. The Group IV (125mg/kg extract + gentamicin) showed a congested central vein, degenerated hepatocytes, and periportal chronic inflammatory cells.



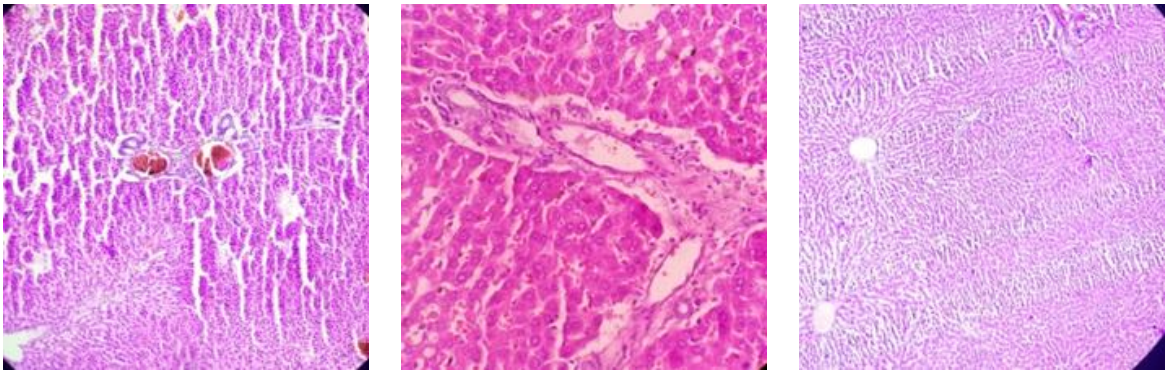
Negative Control (Distilled water)



Ascorbic acid (200 mg/kg) and Genta (80 mg/kg)



Genta (80mg/kg)



500mg/kg Extract and Genta (80mg/kg) | 250 mg/kg Extract and Genta (80mg/kg) | 125 mg/kg Extract and Genta (80mg/kg

Figure 2. Effect of on liver histomorphology in various group

The results are further supported by the observations made in Figure 2, where Group I, the negative control group, showed a normal liver architecture with no abnormalities. In contrast, Group II (Gentamicin only) exhibited liver abnormalities such as portal chronic inflammation, bile stasis, and degenerated hepatocytes. However, the treatment groups that received the tested extract or Ascorbic acid along with Gentamicin (Group III, IV, V, and VI) displayed varying degrees of improvement in liver architecture, with mild chronic inflammation and congested blood vessels observed.Many fern species have been found to contain flavonoids such as quercetin and kaempferol, which have been reported to have hepatoprotective roles.(36) (40) These flavonoids have been reported in various fern species including *Drynaria quercifolia*, *Lygodium japonicum*, *Athyrium multidentatum*, and *Polypodium leucotomos*. (36) Flavonoids are known for their antioxidant and anti-inflammatory properties, which contribute to their potential hepatoprotective effects. These compounds may help protect the liver from oxidative stress and reduce inflammation (37) (39) (38).

This species investigation into nephroprotective action is novel, with no previous studies conducted. Our findings provide new insights into its hepatoprotective and nephroprotective potential, offering a valuable contribution to future therapeutic developments in protecting liver and kidney function.

CONCLUSION

To conclude, the finding suggests that gentamicin had detrimental effect on kidney function and induced nephrotoxicity further leading to liver dysfunction. Whereas, ascorbic acid and the extract was shown to have potential renoprotective and hepatoprotective properties. The extract showed a dose-dependent reduction in (urea, creatinine, ALP, AST) biomarkers, kidney weight and showed protective effects against glomerular injury, along with protective effects on the liver, as indicated by improved enzyme levels and mitigated liver abnormalities. These findings highlight the potential of the tested extract and Ascorbic acid as therapeutic interventions to protect against Gentamicin-induced kidney along with distant organ-damage. Further research is needed to understand the underlying mechanisms and potential therapeutic applications of these interventions in renal dysfunction.

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Conflict of Interest

The authors declare that they have no known competing financial intersets or personal relationships that could have appeared to influence the work reported in this paper

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الفحص الكيميائي النباتي وتقييم التأثير المحسن لمستخلص أوراق نبات *Aleuritopteris bicolor* (Roxb.) Fraser.Jenk. على تلف الكلى والكبد في نموذج فئران يعاني من سمية كلوية مستحثة بالجنتاميسين

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ملخص

الخلفية: أنواع السرخس (AB) *aleuritopteris bicolor*، في عائلة Pteridaceae ، تستخدم تقليديًا لشفاء الجروح وعلاج أمراض مختلفة. الهدف: أجريت هذه الدراسة لتقييم إمكانية استخراج أوراق الإيثانول المائية (AB) في التخفيف من السمية الكلوية الناجمة عن الجنتاميسين والسمية الكبدية في الفئران البيضاء. الأساليب والمواد: تم تحقيق هذه الدراسة من خلال إجراء اختبار كيميائي نباتي على أوراق إيثانول AB المائية يستخلص وإدارة مستخلص الإيثانول المائي لأوراق AB شفهيًا وجنتاميسين (80 ملغ/كغ/يوم) داخل الوزراء ، و Liertains و Liver eigh-، و Liver eigh ، يوم. تم استخدام SPSS الإصدار 23 لتحليل البيانات. تم تقديم النتائج على أنها متوسط الانحراف المعياري (ن = 6). تضمن التحليل الإحصائي ANOVA أحادي الاتجاه متبوعًا باختبار اختلاف أقل أهمية.(LSD)

النتائج: من الفحص الكيميائي النباتي ، تم العثور على مستخلص الإيثانول المائي في (AB) *aleuritopteris* ليحتوي على الفلافونويد والفينولات والسابونين والعفص. أظهر اختبار السمية الحاد سلامته حتى 5000 ملغ/كغ. أسفرت إدارة الجنتاميسين) المجموعة (II عن زيادة كبيرة (P <0.001) في اليوريا (154.07 ± 6.22 ملغ/دل) ، الكرياتينين (8.90 ± 0.51 ملغ/دل) ، وحمض اليوريك (3.27 ± 0.74 ملغ/دل) ، مما يشير إلى مجموعة من العزف الكلوي. أدى المعالجة المشتركة مع حمض الأسكوربيك (المجموعة الثالثة) وجرعات متفاوتة من مستخلص أليوريتوبتيريس بيكولور) (AB) المجموعات IV و V و (VI إلى تخفيضات كبيرة في مستويات اليوريا والكرياتينين ، مع جرعة 500 ملغ/كغ من مستخلصات AB التي تظهر أكثر التأثيرات الملحوظة (P <0.001) مقارنةً بمجموعة .Gentamicin-Inly وكشف تحليل التشريح المرضي أن الجنتاميسين تسبب في تنكس أنبوبي ، وتشكيل المصبوب الغروي ، والإصابة الكبيبية ، في حين أن العلاج مع مستخلص ABيقلل من هذه الأضرار. بالإضافة إلى ذلك ، تسبب الجنتاميسين في زيادات كبيرة في ALP في المصل (P <0.001) ، AST (P <0.001)، و ALT (P <0.001) مقارنة مع المجموعة الضابطة. العلاج مع مستخلص AB قلل بشكل كبير من هذه الإنزيمات (P <0.001)مقارنة مع مجموعة الجنتاميسين فقط. وأظهر التحليل النسيجي أن مجموعة الجنتاميسين كان لها التهاب في البوابة وانحطاط خلايا الكبد ، في حين أن مستخلص AB يقلل من هذه التغييرات ، مما يدعم آثارها الوقائية ضد السمية الكلوية والكبدية.

الاستنتاجات: تشير هذه النتائج إلى أن استخراج *Aleuritopteris bicolor*يخفف بشكل فعال من السمية الكلوية الناتجة عن الجنتاميسين والسمية الكبدية في الفئران البيضاء ، مما يدل على إمكانية الاستخدام العلاجي.

الكلمات الدالة: *Aleuritopteris bicolor* :، تحليل الكيمياء النباتية ، تأثيرات الحماية الكلوية ، تأثيرات الكبد ، سمية الجنتاميسين.

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Wound healing Potential of Fucoidan Extracted Microwavically from *Sargassum wightii* Possibly Mediated by Collagen-1 Expression in Vero Cell Line

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ABSTRACT

Background: Fucoidan, a natural macromolecule extracted from *Sargassum wightii*, has shown promise in various therapeutic areas, including anti-tumor, antioxidant, antithrombotic, and wound healing applications. This study explores the wound healing potential of fucoidan derived from *Sargassum wightii* collected from the Gulf of Mannar, Tamil Nadu, India.

Aims and Objectives: This research aims to assess the effectiveness of fucoidan extracted via microwave-assisted extraction (MAE) in promoting wound healing in Vero cells, a line of African green monkey kidney cells. The study also investigates the impact of fucoidan on collagen-1 expression, a critical protein involved in the wound healing process.

Materials and Methods: Fucoidan was extracted using MAE, and its cytotoxicity was evaluated using the Sulforhodamine B (SRB) Assay. The wound healing efficacy was tested through a scratch assay, measuring the closure of wounds over 24 and 48 hours.

Results: The SRB Assay demonstrated that fucoidan did not exhibit cytotoxicity to Vero cells, with an IC50 value of 61.30 µM. The scratch assay revealed wound closure of 46.15% at 24 hours and 76.9% at 48 hours, compared to 50% and 81.25% in the control group. Fucoidan treatment significantly increased collagen-1 expression, with 77.92% of cells showing elevated levels of this crucial protein.

Conclusions: This study confirms the in-vitro wound healing capabilities of fucoidan extracted from *Sargassum wightii*. These findings support the potential of fucoidan as a natural agent for wound healing and restoration.

Keywords: *Sargassum wightii*, Fucoidan, macromolecule, Wound healing, SRB assay, Vero cells, collagen-1 expression.

1. BACKGROUND

The world's oceans are teeming with a diverse array of multicellular marine plants known as seaweeds, or macroalgae. These marine plants are categorized into three primary groups based on color: red, green, and brown algae [1]. Seaweed has been a dietary staple in Asia for millennia, highlighting its importance as a food source [2]. Among these, the brown algae genus Sargassum thrives in warm, subtropical oceans, forming vast floating islands known as Sargasso Seas. These islands, consisting of dense mats of Sargassum, can travel extensive distances via ocean currents. Sargassum (figure1) has been utilized in various applications, including food, traditional medicine, and fertilizers [3]. However, further research is essential to fully understand its biological functions and optimize its extraction and utilization methods [4].

Brown seaweeds, such as species from the genera

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Fucus, Undaria, and Laminaria, contain a complex polymer called fucoidan. This sulfated polysaccharide consists of repeating units of glucuronic acid, fucose, and sulfate residues [5]. Fucoidans are believed to interact with various growth factors, such as basic fibroblast growth factor (bFGF) and remodeling growth factor, potentially through heparin-binding interactions, to exert beneficial effects. The chemical structure of fucoidan (figure 2) is highly variable and complex, depending on the source seaweed, extraction method, and environmental conditions. Typically, it comprises a backbone of α-(1→3)- and α-(1→4)-linked L-fucose residues, with branches of sulfate groups and occasional acetyl groups. This structural diversity contributes to its broad spectrum of biological activities [6]. Reducing the molecular weight of fucoidans is thought to enhance their bioactivity, as high molecular weight fucoidans (HMF) are less absorbable and exhibit low tissue absorption [7]. The chemical composition and quantity of fucoidan vary depending on the source seaweed, making it a promising candidate for developing novel therapeutics for various diseases. Yet, comprehensive studies are necessary to elucidate its mechanisms of action and therapeutic potential [8].

Extraction techniques, such as solvent extraction, isolate target compounds from complex mixtures or botanical sources based on their physical or chemical properties [9]. Microwave-assisted extraction (MAE) has successfully extracted numerous biologically active substances from natural sources. This technique uses microwave energy to induce molecular friction through the dipolar rotation of polar molecules, resulting in a volumetrically distributed heat source. MAE often achieves faster, more selective extraction with higher yields and significantly reduced energy and solvent consumption, making it environmentally friendly [10].

The purification process involves removing contaminants to produce a high-quality product. Ion exchange chromatography is a chromatographic method used to separate and purify crude chemicals. Resins like DEAE Sephadex A-25 are commonly used in biochemistry and molecular biology for protein purification and separation [11]. Fucoidan's complex and variable nature makes it challenging to describe its chemical structure using a single method. Techniques such as mass spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and nuclear magnetic resonance (NMR) are employed to gain a deeper understanding of its composition [12].

The Sulforhodamine B (SRB) assay is a colorimetric method used to measure cell death and growth, frequently applied in drug development, pharmacology, and cancer research to assess drug effects on cell viability. Cytotoxicity refers to the capacity of various substances, including chemicals, drugs, or environmental pollutants, to harm or kill cells. It is typically assessed in vitro by evaluating changes in cell viability or metabolic activity following exposure to the substance [13].

Developed in the 1960s, the Vero cell line originates from the kidney tissue of the African green monkey. These adherent cells grow as a monolayer on culture tube surfaces, exhibiting fibroblast-like morphology. Vero cells are known for their rapid growth and ability to form a dense cell layer. Cryopreserved at -80°C in glycerol or dimethyl sulfoxide, Vero cells are utilized for drug screening and toxicity testing to understand the cytotoxic effects of potential medicinal compounds [14].

A wound, encompassing injuries such as cuts, scrapes, punctures, burns, or ulcers, disrupts the normal structure and function of skin or tissue, leading to pain, inflammation, infection, and delayed healing [15]. The scratch test, also known as the wound repair assay, is a laboratory method used to observe cell migration. A scratch is made on a cell monolayer, and the closure of this scratch over time is monitored to study cell migration and wound healing [16].

Recent studies have explored the therapeutic potential of marine-derived compounds, particularly in wound healing applications. Fucoidan from various seaweed

species has demonstrated anti-inflammatory, antioxidant, and antimicrobial properties, making it a promising candidate for wound healing treatments [17]. Moreover, fucoidan's ability to enhance collagen production and promote fibroblast migration has been documented, highlighting its potential in tissue regeneration and repair [18].

This study investigates the wound-healing properties of fucoidan derived from Sargassum wightii by evaluating fibroblast migration in an in vitro scratch assay and measuring collagen-1 levels, an essential extracellular matrix component. The findings could pave the way for the development of new, fucoidan-based therapies for improved wound management and skin regeneration [19, 20].

To advance the application of fucoidan in biomedical fields, it is crucial to establish efficient extraction, purification, and characterization protocols. Integrating advanced techniques such as microwave-assisted extraction and ion exchange chromatography can optimize the yield and purity of fucoidan, ensuring its efficacy in therapeutic applications. Additionally, comprehensive in vitro and in vivo studies are necessary to fully understand fucoidan's bioactivities and mechanisms of action, providing a robust foundation for its clinical use [21, 22].

Figure 1: Image of *Sargassum wightii*

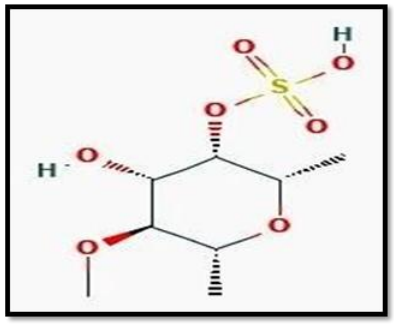

Figure 2: ChemicalStructure of Fucoidan

2. MATERIALS AND METHODS

Materials

The Vero cells (kidney fibroblasts from African green monkeys) were procured from the National Centre for Cell Science (NCCS) in Pune. All chemicals and products used in the study were sourced from Research Lab Fine Chem, Inc., including antibiotics, FBS (from PAA Labs, catalog number A11-043), and DMEM (Dulbecco's Modified Eagle's Medium) serum.

The study utilized various instruments: an FTIR Hyperion 3000 from Bruker (Germany), an NMR ECZR series 600MHz from Jeol (Japan), a mass spectrometer

Trift V nano TOF from Physical Electronics (USA), DEAE Sephadex A-25 from GE Healthcare (USA), a 96-well tissue culture plate from Corning (catalog number 3599), an inverted microscope from TMS (Nikon), and a microwave MSD-2000 from CEM Corporation (Matthews, NC).

Both the SRB Assay and the Scratch Assay were conducted at Aakaar Biotechnologies Pvt Ltd in Lucknow.

Methods

Source of Fucoidan

The freshly harvested Sargassum wightii came from Mandapam on the Indian coast, in the Gulf of Mannar. To get rid of any physical contaminants, like mud and particle matter stuck to the plant, it was completely washed. It was dried under shadow at room temperature for 10 to 15 days.

Authentication

The Regional Facility for DNA Fingerprinting, Thiruvananthapuram, Kerala, India's PG Department of Botany and Research Centre of Rajiv Gandhi Centre for Biotechnology authenticated and correctly identified the *Sargassum wightii* member of the *Sargassaceae* family by referring to a herbarium.

Extraction of fucoidan by Microwave Assisted Extraction (MAE)

Microwave-assisted extraction (MAE) has been a well-established method for processing plant materials [23, 24]. The procedure involves placing reaction tanks, connected by tubes, into a rotating carousel within the sample holder. One of the tubes is equipped with a pressure monitor to track and maintain the set pressure. The extracted fucoidan from *Sargassum wightii* has demonstrated potential in promoting wound healing, as depicted by the Figure 1.

An equation was used to figure out the fucoidan extraction yield (% F) after the extraction was helped by microwaves. WMOHis the dry mass weight that was found after ethanol precipitation. WA is the weight of the algae that was used in each trial.

%F = WMOH/WA × 100

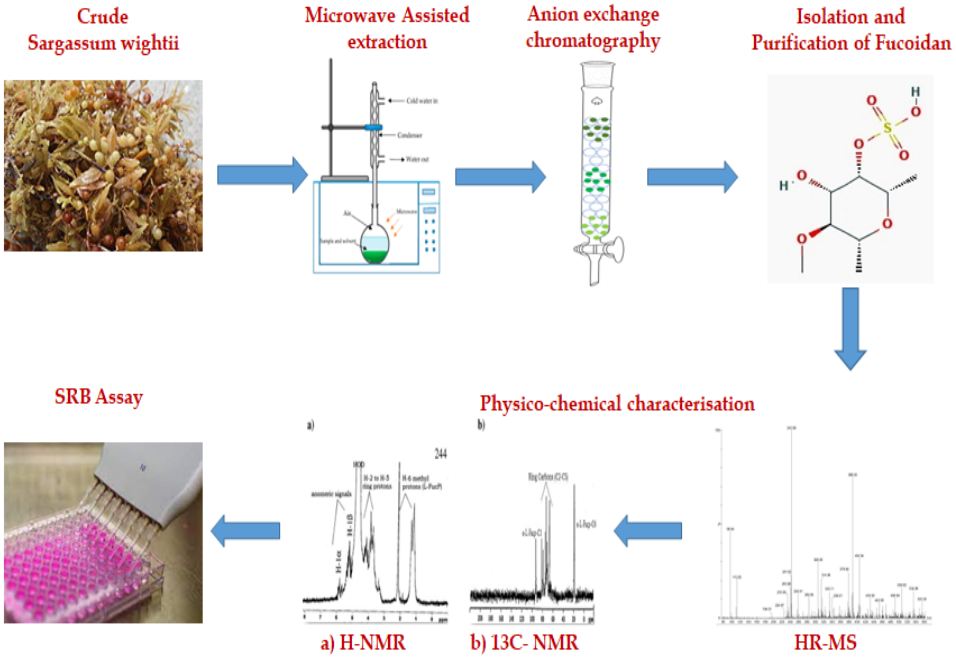

Figure 3: Illustrative depiction of the extraction and characterization process of fucoidan obtained from *Sargassum wightii*

Fucoidan Purification Using Anion Exchange Chromatography

The fucoidan extracted and precipitated with ethanol underwent further purification through anion-exchange chromatography (AEC) [25, 26]. To eliminate proteins and uronic acids, calcium chloride was added, followed by isoelectric point precipitation. Figure 4 presents a flow chart detailing the purification process of fucoidan from *Sargassum wightii*.

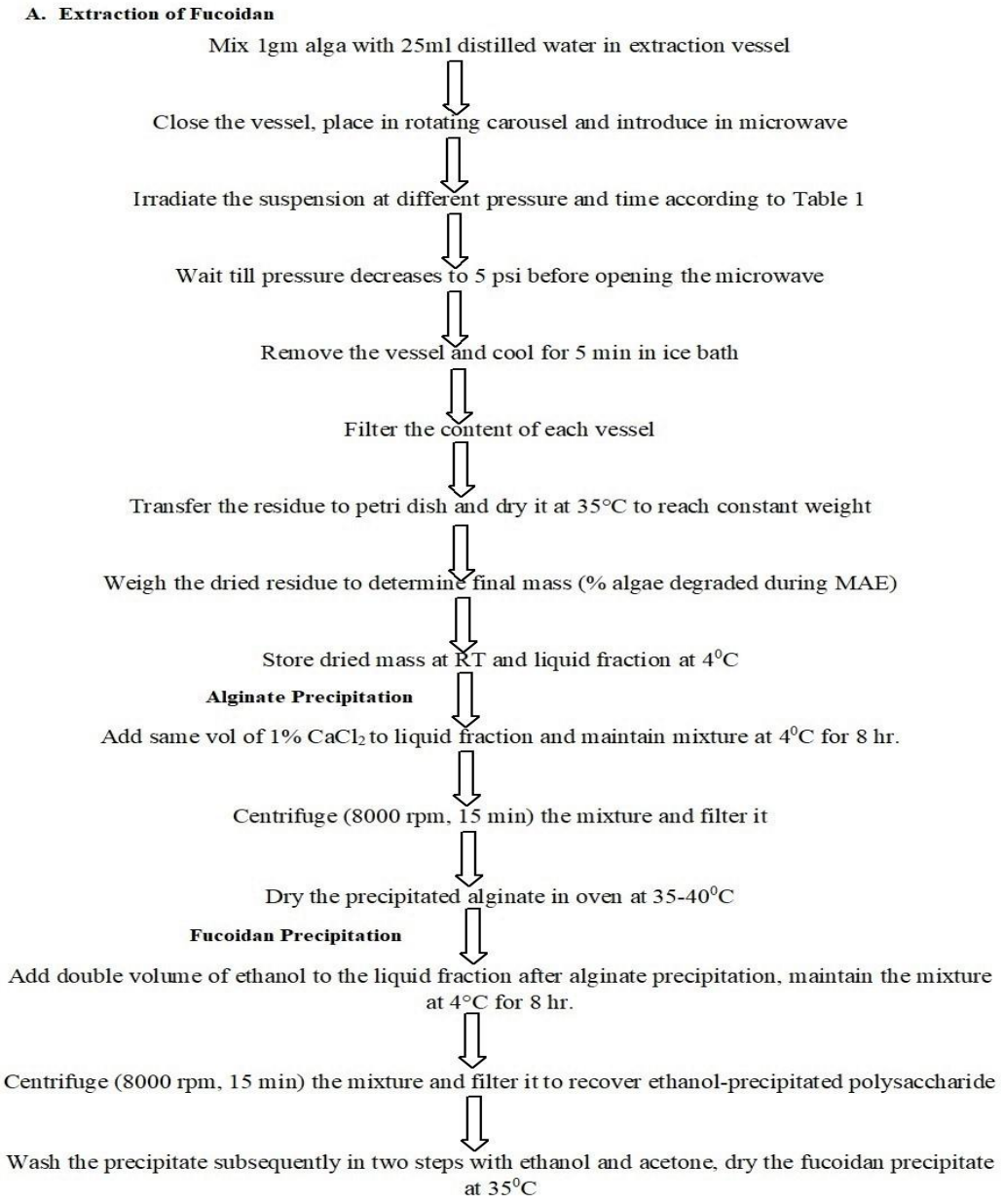

Fig 4: Flow chart presenting A) Extraction of fucoidan from *Sargassum wightii*

B. Purification of fucoidan

Protein Precipitation

Isoelectric point precipitation: Add HCL acid from pH 6-3 to fucoidan until isoelectric point is reached. Incubated the solution of pH 3 at RT for 4 hrs.

⇩

Salting out: Add ammonium sulphate to final concentration of 80% and incubate at 4^0C for 12 hrs

⇩

Trichloroacetic acid (TCA) denaturation: Add dilute TCA solution to final concentration of 10%, incubate at RT for 4 hrs and centrifuge the resulting solution at 6000rpm for 3 min to precipitate proteins

⇩

Uronic Acid precipitation

To the supernatant add different concentrations (1,2,3 and 4%) of calcium chloride, incubate at 4^0C for 4 hrs and centrifuge at 6000rpm for 3 min to precipitate uronic acid

⇩

The supernatant was then filtered, and lyophilized for further purification through anion-exchange chromatography

⇩

After protein and uronic acid precipitation 300mg of fucoidan was dissolved in distilled water at a concentration of 10 mg/mL and applied to DEAE Sephadex A-25 column

⇩

After loading with fucoidan, elution was performed using a NaCl gradient with four different NaCl concentrations (1, 2, 3, and 4 M) at a flow rate of 1 mL/min.

⇩

. Fractions containing fucoidan were collected, filtered, and lyophilized to obtain purified fucoidan.

⇩

The total carbohydrate content of each tube was measured at 490 nm by the phenol–sulfuric acid colorimetric method

Fig 4: Flow chart presenting B) Purification of fucoidan

Characterization of fucoidan

Fourier Transform Infra-Red Spectroscopy (FTIR)

To analyze the infrared spectra of fucoidan samples, we utilized Fourier transform infrared spectroscopy (FTIR) on a Bruker Hyperion 3000 with Vertex 80, carried out at SAIF IIT Bombay, Powai, Mumbai. For this analysis, we created pellets by blending about 1 mg of the fucoidan sample with 100 mg of KBr salt in a 1:100 ratio and pressing them into flakes using a hydraulic pellet press. This method enabled us to capture the sample spectra across the range of 400 to 4000 cm^{-1} with a precision of 4 cm^{-1}. We then meticulously examined the spectra to identify possible linkage types and functional groups [27, 28].

Nuclear Magnetic Resonance Spectroscopy (NMR)

The fucoidan fraction was dissolved in D2O at 10 mg/mL for 2D NMR analysis [27, 28]. This was performed at 300 K using a 500 MHz Bruker Avance DRX spectrometer with a TXI 5 mm probe. The HSQC pulse sequence from Bruker's database was used, and spectra were recorded at 360/120 ppm for 1H and 1024/9 ppm for 13C [27, 28].

Q-TOF Mass spectroscopy (MS)

Mass spectrometry was employed to analyze the Fucoidan substance. The Xevo G2-XS Q-TOF, manufactured by Waters Corporation USA [31–33], was replicated. The mass spectrometer may generate negative-ion mode results within the m/z range of 100 to 3000. The capillary was supplied with a power of 3 kilovolts, while the cone received a power of 45 volts. The gas desolvation rate was adjusted to 800 liters per hour, while the cone gas flow rate was set to 50 liters per hour. The samples were introduced at a flow rate of 10 µl/min after being mixed with methanol and filtered.

SRB Assay

The samples were evaluated for their cytotoxicity on the Vero cell line (kidney fibroblasts from African green monkeys) [34–36]. Vero cells were cultured in a 96-well plate at 37°C with 5% CO2 in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin antibiotic for 24 hours. The following day, cells were treated with varying concentrations of fucoidan (1–1000 µM).

For the cytotoxicity assessment, the Sulforhodamine B (SRB) assay was used. After 24 hours of fucoidan treatment, 100 µL of 10% Trichloroacetic Acid (TCA) was added to each well to fix the cells. The plate was washed with deionized water and air-dried. Subsequently, 0.04% SRB solution was added to each well and incubated for one hour to stain the proteins. Excess dye was removed by washing with 1% acetic acid, and the plate was dried at room temperature. The bound dye was solubilized with a tris base solution (pH 10.5) and mixed on an orbital shaker for 10 minutes. The absorbance was measured at 510 nm using an Elisa plate reader (iMark, Biorad, USA).

Scratch Assay

The wound healing capabilities of the fucoidan extract were assessed through in vitro cell migration studies on Vero cells. Initially, 10,000 Vero cells per well were cultured in a 96-well plate for 24 hours in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO2. On the following day, a scratch was made in the cell monolayer using a 200 µL pipette tip. The cells were then treated with 51.6 µg/mL of fucoidan extract and 5 µg/mL of povidone-iodine (positive control) and incubated for 24 hours.

Cell migration and morphological changes were captured using an inverted microscope equipped with a digital camera. The width of the scratch and wound closure at 0, 24, and 48 hours were analyzed using ImageJ software (NCBI) and presented graphically. The wound area percentage, A(t), was tracked to quantify cell migration indirectly:

$$A(t) = A(t)/A(0) \times 100\%$$

where A(t) is the wound area at time t and A(0) is the initial wound area.This method allows for the indirect evaluation of the migration rate by measuring the percentage of wound area at specific time points.

Expression Studies by Flow Cytometry

Flow cytometry [39, 40] was used by researchers to investigate the impact of fucoidan on collagen 1, a molecule involved in wound healing. Inoculate a 6-well microtiter plate with a density of 2 x 106 Vero cells/mL and allow them to proliferate overnight. The cells were exposed to a concentration of 125 ng/mL of plant extract and 10 ng/mL of positive control hEGF for a duration of 48 hours. Rinsing with DPBS following trypsinization to disperse cells. The cells were preserved in 70% methanol at a temperature of -20°C for an extended period of time to analyze the expression of collagen 1. The cells were labeled with an anticollagen 1 antibody and rinsed with DPBS. Subsequently, they endured a 30-minute period of darkness under ambient conditions. Fluorescence-based cell sorting Protein expressions were evaluated using Calibur flow cytometry, and the findings were analyzed using CellQuest Pro software.

Statistical Analysis

The results are shown as the mean percentage inhibition with standard deviation (SD) (n = 3). Each experiment was done three times. A one-way analysis of variance was used to find statistical significance. A value of $p < 0.05$ was considered statistically significant. After this, the Bonferroni post hoc test for multiple comparisons was done. All statistical tests and IC50 value estimates were done with GraphPad Prism (version 3.1) software.

3. RESULTS

Extraction of fucoidan

Through the optimization of microwave-assisted extraction parameters, the yield of fucoidan from *Sargassum wightii* was significantly enhanced. The highest yield, 88.22%, was achieved under conditions of 120 psi pressure for 1 minute and an algal-to-solvent ratio of 1:25 gm/ml. Purification of Fucoidan

The study explored the impact of varying NaCl concentrations (1, 2, 3, and 4 M) on the elution of fucoidan. It was found that using 1 M NaCl resulted in the highest total sugar content, but the lowest sugar recovery [26]. When 2 M NaCl was employed, the total sugar content decreased slightly, but the recovery efficiency significantly improved, reaching nearly 76%. Although higher NaCl concentrations (3 M and above) made sugar recovery easier, they resulted in much lower total sugar content. These findings demonstrated that 2 M NaCl was the optimal concentration for fucoidan elution, as stronger NaCl solutions did not enhance purity (Table 1).

Consequently, 2 M NaCl was chosen as the eluent for anion exchange chromatography (AEC). The implementation of microwave-assisted extraction (MAE) markedly increased the purity of total sugar extracted from *Sargassum wightii*. The purity soared from 6.94% to 64.5%, with a fucoidan carbohydrate recovery rate of 50.3% ± 3.5%. Following these advanced processing steps, the total sugar content reached 70.5 ± 2.56%, and the sulfate group content was 19.1 ± 0.58%. These results indicate that the fucoidan achieved an impressive purity level close to 90%, showcasing the efficacy of this extraction and purification method (Figure 5).

Table 1: Purification of Fucoidan Using Anion Exchange Chromatography

Parameters	**Control**	**NaCl concentration (Molar)**			
		1 M	2 M	3 M	4 M
Total sugar content (% of dry weight)	53.75	54.6	69.87	70.19	64.24
Sugar recovery (%)	100	51.08	77.09	80.93	82.65
Total sugar content after dialysis cut off (% of dry weight)	**47.3**	**48.97**	**64.95**	**55.74**	**56.95**

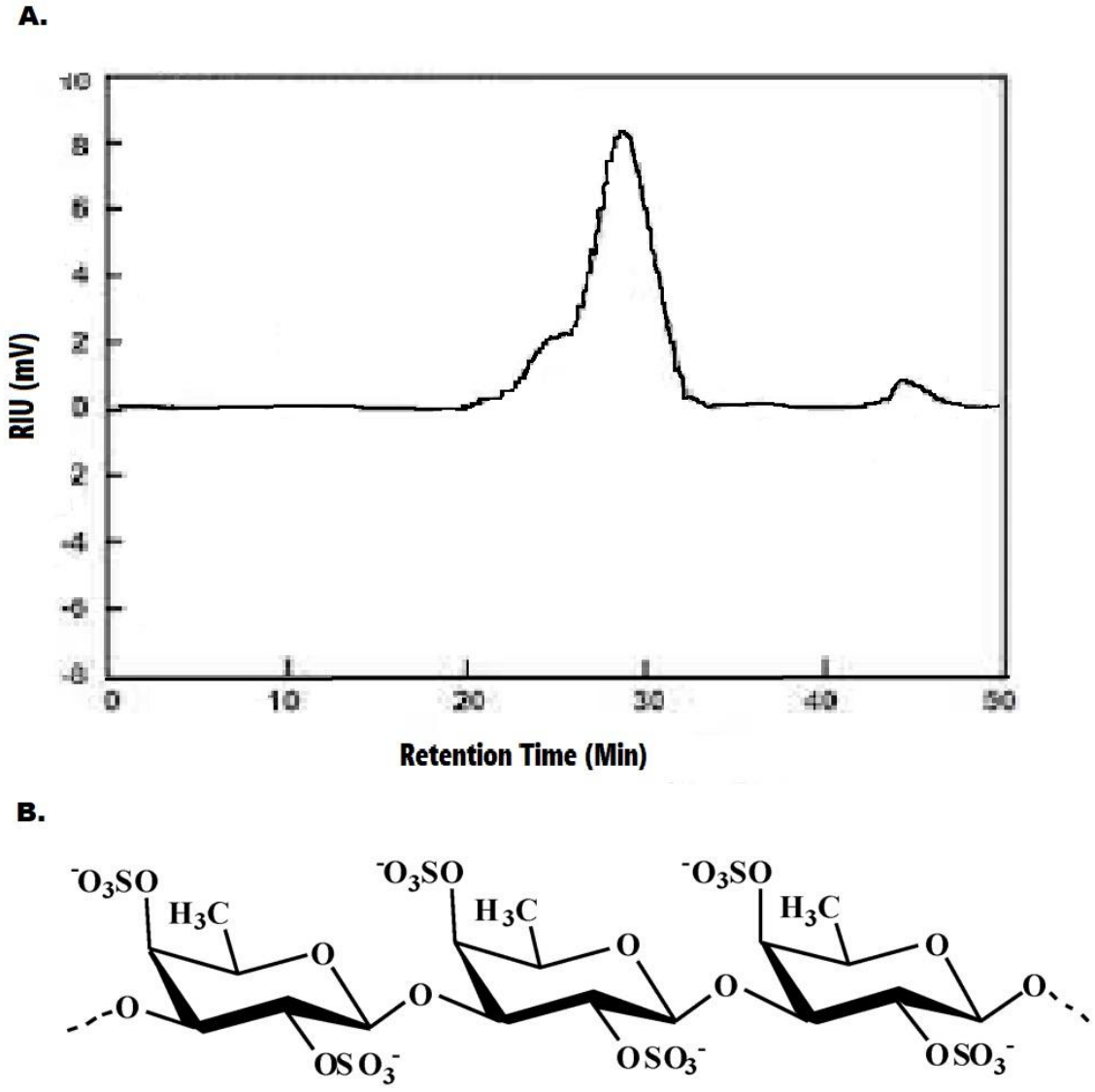

Fig 5: AEC Chromatogram of fucoidan separation using DEAE column

Characterisation of Fucoidan

FTIR analysis

The purpose of the FTIR investigation was to identify infrared absorption characteristics indicative of fucoidan [28]. The FTIR spectra (Figure 6) revealed distinctive fucoidan bands at 1256.6 and 1258.8 cm^{-1}. Absorption within this range indicates the presence of sulfates, corresponding to the asymmetric O=S=O stretching vibrations of sulfate esters. Additionally, an IR band at 839 cm^{-1} indicates the COS bending vibration of sulfate substituents at position C4. This suggests a strong sulfation pattern at the C4 position of the monosaccharide in the polysaccharide chain.

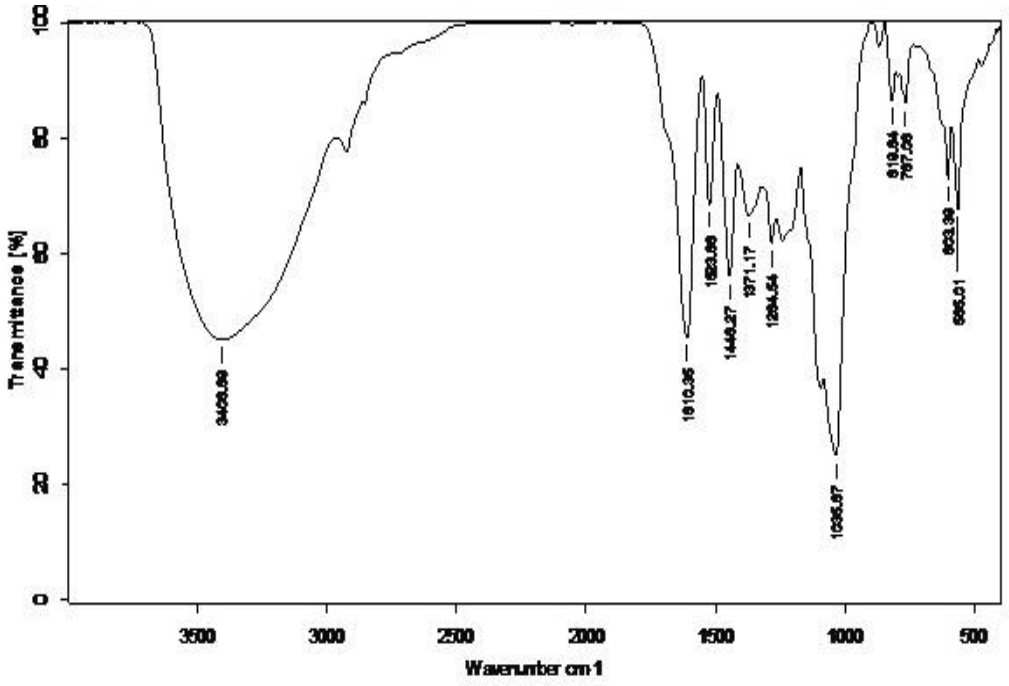

Fig 6: FTIR spectra of fucoidan from *Sargassum wightii*

NMR Analysis

To ensure the accuracy of these structures [30], we analyzed fucoidans using **H^1 and C^{13}** NMR spectra (Figure 7). The key for the labeled peaks can be found in Table 2.

Table 2: Assignment of chemical shift of H^1 and C^{13} NMR spectra of fucoidan extracted from *sargassum wightii*

Sr.No	Hydrogen and carbon of functional groups	Signal of H^1 proton	Signal of C^{13}
1.	H-1[α] = protons that are α-anomeric	5.0–5.1	90–105
2.	H-1[β] = protons that are β-anomeric	4.24 – 5.0	101 – 109
3.	H-(2/3/4) [O-Ac] = protons needed for O-acetylation at sites C2, C3, and C4	4.88, 4.97, 4.98, 5.09, 5.12	70.7, 71.0, 70.0, 70.2, 71.1
4.	[O-sulfate] (H-(2/3/4)) = protons connected to O-sulfation at places C2, C3, and C4	4.5– 5.0	75–85
5.	H-6[CH2] = protons of hexose -CH2OH	3.24, 3.90	67.39
6.	H-6[CH3] = fucose of O-Ac [CH3] = acetyl CH3 protons	5.25, 5.50	67.4, 69.1

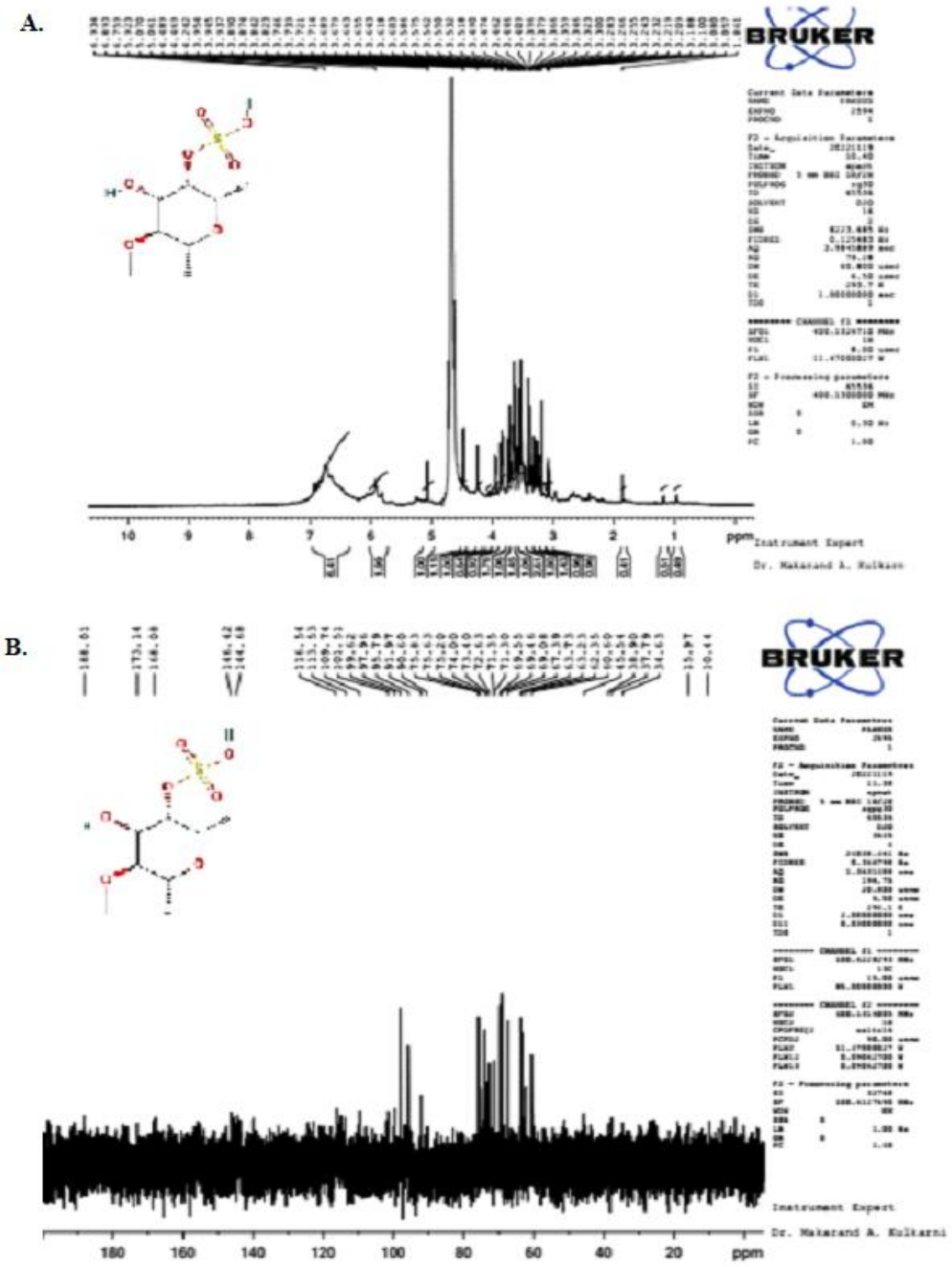

Fig 7: A) H^1 NMR and B) C^{13} NMR Spectrum of Fucoidan isolated from *Sargassum wightii*

Mass analysis

The structure of fucoidan is better understood by using Q-TOF for mass studies [32, 33]. Hexoses (m/z 259), desoxyhexoses (m/z 243), and probably pentoses (m/z 229) all go through sulfation at C-2, which makes the cross-ring cleavage at m/z 138.9 send out a strong signal. Another sign that shows the sulfation at C-2 is at m/z [M-18]. It is possible to separate the C-4 and C-6 sulfations on hexoses to make a fragment ion with a mass of 198.9. The breaking apart of the ring-opening ion at m/z 168.9 points to sulfation at C-3. There is a strong signal at m/z 225, which means that the fucose residue on the non-reducing end is sulphated at position C-2. The sulphate is close to the glycosidic bond, which makes it easier to cut. Too much desulfation broke down the α-L-fucan, which was initially more strongly sulfated (3-linked and 2,4-disulfated). However, its main part (2,4-disulfate of α-L-Fuc p) and the 3-linked fuco-oligosaccharides of the main chain could still be seen clearly by Q-TOF-MS, as shown in figure 8.

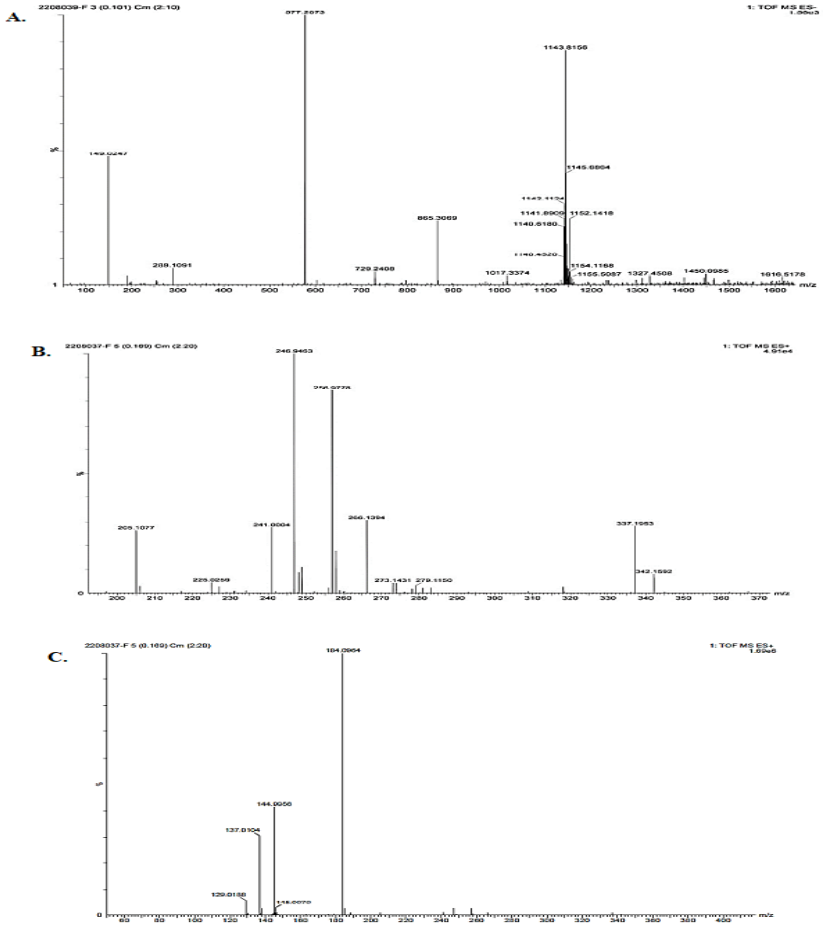

Fig 8: Q-TOF mass spectra of sulphated polysaccharide-Fucoidan from *Sargassum wightii*

SRB assay

To calculate the IC50 [34-36], we plotted the dose-response curve, showing cell viability at various fucoidan concentrations. We identified the point where cell viability was 50% of the maximum (125%). From this point on the y-axis, we drew a line intersecting the dose-response curve, then extended a vertical line down to the x-axis to find the corresponding concentration.

The IC50 value was determined to be 61.3 µM, as shown in Figure 9. This approach ensures the IC50 accurately represents the concentration required to achieve a 50% reduction in cell viability from the peak response.

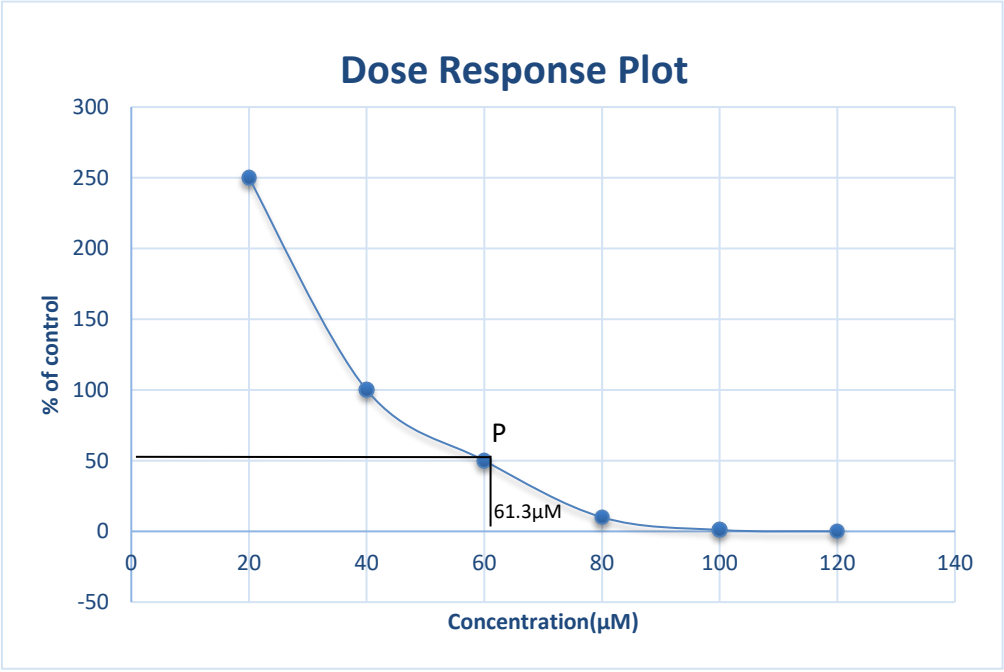

Fig 9: Graphic Determination of IC50 from dose response plots.

Scratch assay

The migration rate was directly evaluated as the percentage of wound area healed at specific time points [37-39]. For the control group treated with 1% povidone-iodine, the wound area healed was 43.75% at 24 hours and 81.25% at 48 hours. For the test group treated with fucoidan, the wound area healed was 46.15% at 24 hours and 76.92% at 48 hours. The statistical analysis yielded a p-value of 0.05, indicating that there is no statistically significant difference between the wound healing rates of the fucoidan-treated group and the control group. This suggests that fucoidan exhibits wound healing properties comparable to those of the control treatment.

The wound healing process was monitored using the ImageJ program to measure the scratch width at 0, 12, 24, and 48 hours. The experiments were performed in triplicate, and the results confirmed that fucoidan facilitated faster wound healing than the control group, as shown in Figure 10.

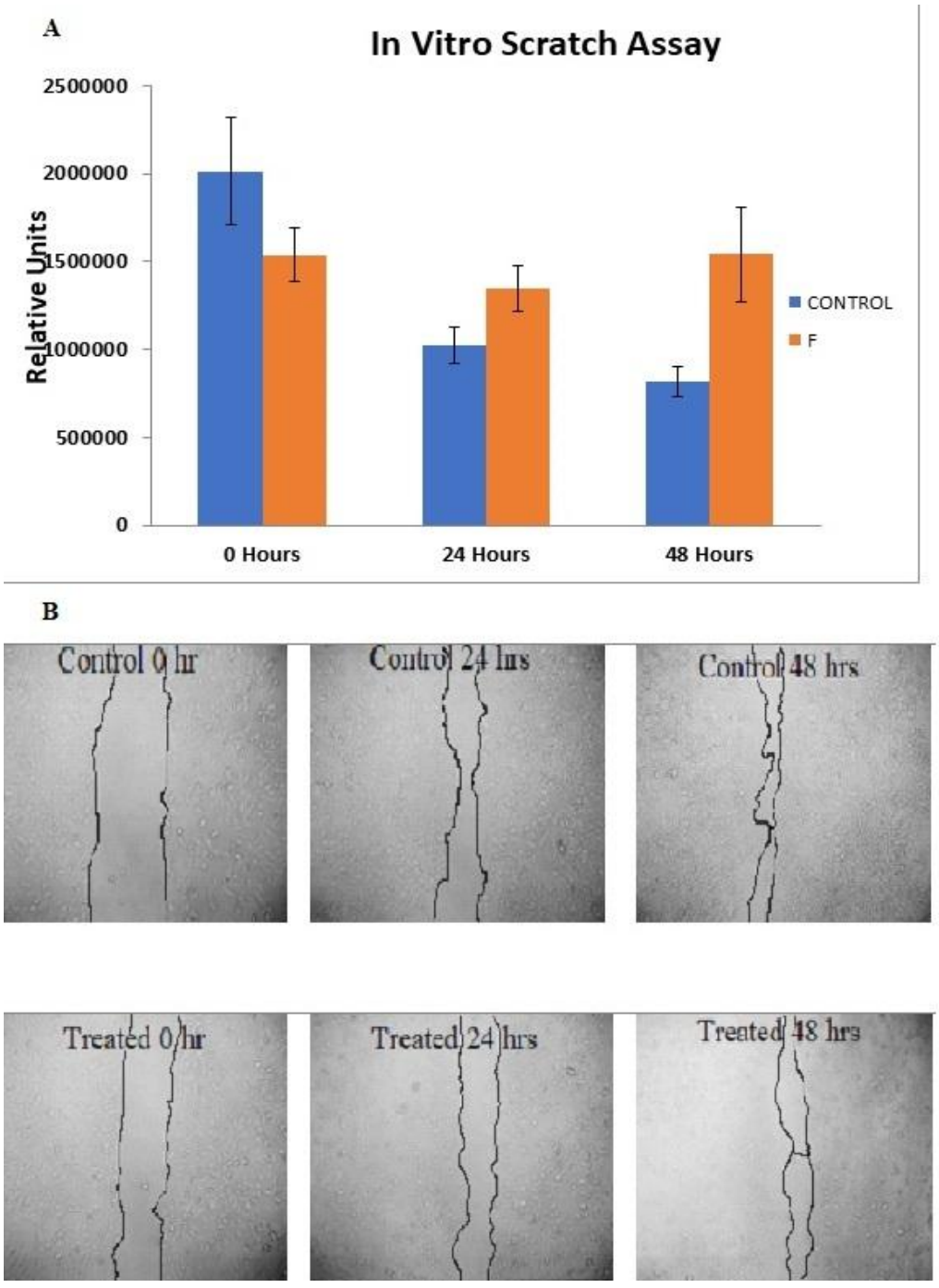

Fig 10: Time-lapse images of scratch assay

Collagen-1 expression

One of the main proteins in the extracellular matrix (ECM) is collagen-1. It helps make the ECM during the mending process and also encourages the growth, migration, differentiation, and production of other important proteins from skin cells [41, 42]. This study looked at how much Collagen type 1 was expressed in vero cells 48 hours after they were treated with either 125 µg/mL of fucoidan extract or 10 ng/mL of human Epidermal Growth Factor (hEGF). The experiments showed that cells treated with fucoidan and hEGF had higher levels of collagen type 1. In cells treated with extract, 77.92% of cells showed collagen type 1, and in cells treated with hEGF, 90.10% of cells did. Figure 11 shows the flow cytometric study of Collagen type 1 expression and the number of expressing cells in cells that have not been treated, cells that have been treated with extract, and cells that have been treated with hEGF. The results show that the extract clearly raises the expression of Collagen type 1 in cells treated with it compared to the control group that wasn't treated. This suggests that the extract raises the expression of Collagen type 1 in vero cells, which may help the wound heal.

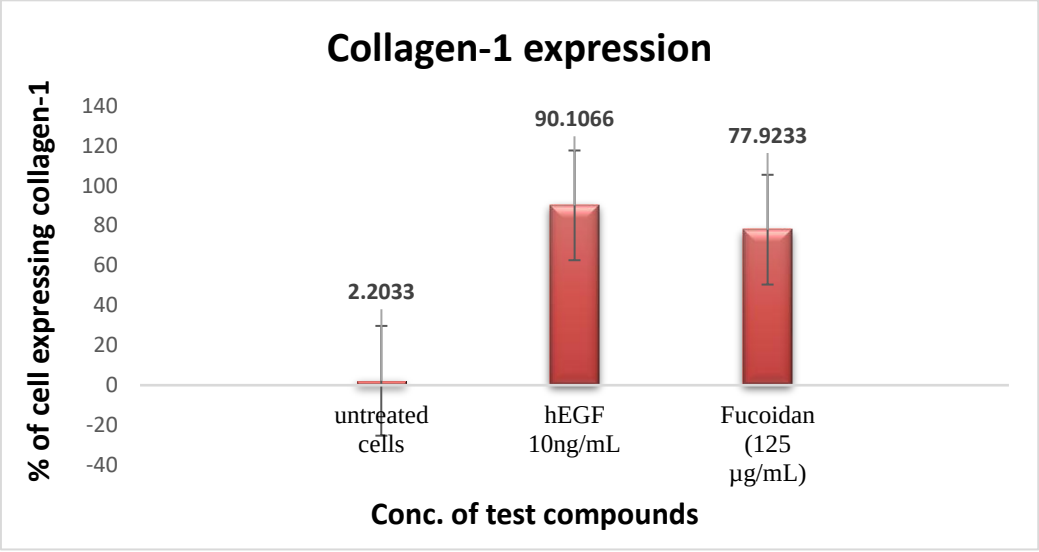

Fig 11: Bar graphs represent the % of Vero cells that expressed collagen-1 after being treated for 48 hours with 125 µg/ml of fucoidan and 10 ng/ml of hEGF

4. DISCUSSION

To thoroughly evaluate the wound healing efficacy of microwave-extracted fucoidan from **Sargassum wightii**, we performed scratch assays and analyzed collagen I expression levels. Our findings provide strong evidence that the extracted fucoidan significantly promotes fibroblast migration and proliferation—two critical processes essential for effective wound closure. These cellular activities are closely associated with the activation of key signaling molecules such as VEGF, PDGF-BB, and collagen, which play vital roles in epithelial regeneration, angiogenesis, and extracellular matrix (ECM) remodeling.

Collagen I, which constitutes approximately 70% of the body's collagen, is a major structural component of the ECM and plays a pivotal role in the wound healing process. The accelerated synthesis of connective tissue proteins, particularly collagen I, is indicative of active ECM formation and restructuring, which are essential for the

repair and regeneration of damaged tissue. The interaction between fibronectin and collagen is another crucial factor that enhances wound healing, as it supports the stabilization and integrity of the newly formed ECM [40].

The final phase of wound healing, tissue remodeling, is characterized by scar formation and the restructuring of the ECM, a process that further underscores the importance of collagen I and fibronectin. An increase in the levels of these proteins is a strong indicator of the wound healing potential of the materials tested in this study.

To further understand the role of fucoidan in wound healing, we analyzed collagen I expression in Vero cells treated with human epidermal growth factor (hEGF) as a reference, fucoidan extract, and a control. The results revealed a significant increase in collagen I expression in fucoidan-treated Vero cells, which was comparable to the hEGF-treated control. These findings suggest that fucoidan enhances wound healing by promoting fibroblast migration and proliferation, a process facilitated by the upregulation of collagen I. This aligns with previous studies that have demonstrated elevated fibronectin expression when plant polyphenols are utilized for wound healing [42-46].

The implications of these findings are significant, as they suggest that the fucoidan extracted from **Sargassum wightii** is not only effective in promoting wound healing but also non-toxic, making it a promising candidate for further research and potential therapeutic applications. This study is particularly noteworthy as it represents the first in vitro demonstration of the wound healing potential of fucoidan derived from **Sargassum wightii**. Further studies, including in vivo analyses, are warranted to explore the full therapeutic potential of this natural compound in wound care and tissue regeneration.

CONCLUSION

Fucoidan from Sargassum wightii can be recovered through MAE in the best response conditions. The fast extraction time and use of chemicals that don't damage the material kept costs down. It was suggested that MAE could be used to get fucoidan from brown kelp. The FTIR and 2D NMR results show that the fucoidan found in Sargassum wightii is made up of a fucose-galactose backbone that is linked by 1,3 glycosidic links and has sulfation at C2 and C4 sites. The fucoidan water mix helped the Vero cell line heal wounds faster. Furthermore, it was demonstrated that the extract did not have any damage to cells. Based on these findings, fucoidan may be a good source of natural chemicals that can help heal wounds.

In our study, we show that increased collagen 1 expression and fibroblast movement to the wound site affect fucoidan's ability to heal wounds. In addition, this extract does not harm cells and is safe for further study. According to these results, fucoidan may help wounds heal, and more research needs to be done to find the substances that make this happen.

Declarations

Ethics permission: The Ethics Committee has confirmed that there is no need for ethical permission.

Permission to publish: The writers have given permission to publish.

Data Access: All the data that was collected or analyzed for this study is in this published piece and the files that go with it.

Competing Interests: The authors of this piece do not have any interests that are at odds with what it says.

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Contributions of Authors: All of the authors helped come up with the idea for and plan the study. (DR. Smita, Tukaram Kumbhar), (DR. Manish, Sudesh Bhatia), and (MS. Shubhangi, M. Birajdar) prepared the materials, collected the data, and analyzed it. The manuscript's first draft was written by (DR. Smita, Tukaram Kumbhar), and all of the authors gave feedback on earlier drafts. The final manuscript was read and agreed upon by all the writers.

List of abbreviations:

MAE: Microwave-assisted extraction
bFGF: basic fibroblast growth factor
HMF: High molecular weight fucoidans
SRB: Sulforhodamine B
ECM: Extracellular matrix
DMEM: Dulbecco's Modified Eagles Medium
FBS: Fetal Bovine Serum
PBS: Phosphate Buffered Saline
FTIR: Fourier-transform infrared
NMR: Nuclear magnetic resonance
MS: Mass spectroscopy
AEC: Anion-exchange chromatography
HSQC: Heteronuclear single-quantum coherence

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إمكانية التئام الجروح باستخدام الفوكويدان المستخرج بالموجات الدقيقة من نبات سارجاسوم وايتي، ربما بوساطة التعبير عن الكولاجين-1 في سلالة خلايا فيرو

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ملخص

الخلفية: أظهر الفوكويدان، وهو جزيء طبيعي كبير مستخلص من نبات سارجاسوم وايتي، نتائج واعدة في مجالات علاجية متنوعة، بما في ذلك تطبيقات مضادة للأورام، ومضادة للأكسدة، ومضادة للتخثر، ومضادة للجروح. تستكشف هذه الدراسة إمكانات الفوكويدان المستخلص من نبات سارجاسوم وايتي، المُجمع من خليج مانار، تاميل نادو، الهند، في التئام الجروح.

الأهداف: يهدف هذا البحث إلى تقييم فعالية الفوكويدان المستخلص بتقنية الاستخلاص بمساعدة الميكروويف (MAE)في تعزيز التئام الجروح في خلايا فيرو، وهي سلالة من خلايا كلى القرد الأخضر الأفريقي. كما تبحث الدراسة في تأثير الفوكويدان على التعبير الجيني لبروتين الكولاجين-1، وهو بروتين أساسي يشارك في عملية التئام الجروح.

النتائج: أظهر اختبار SRB أن الفوكويدان لم يُظهر سمية خلوية لخلايا فيرو، حيث بلغت قيمة IC50 61.30 ميكرومولار. وكشف اختبار الخدش عن إغلاق جرح بنسبة 46.15% بعد 24 ساعة و76.9% بعد 48 ساعة، مقارنةً بـ 50% و81.25% في المجموعة الضابطة. وقد أدى علاج الفوكويدان إلى زيادة ملحوظة في التعبير عن الكولاجين-1، حيث أظهرت 77.92% من الخلايا مستويات مرتفعة من هذا البروتين الحيوي.

الخلاصة: تؤكد هذه الدراسة قدرة الفوكويدان المستخلص من نبات سارجاسوم وايتي على التئام الجروح في المختبر. وتدعم هذه النتائج إمكانات الفوكويدان كعامل طبيعي لالتئام الجروح وترميمها.

الكلمات الدالة: Sargassum wightii :، Fucoidan، جزيء كبير، التئام الجروح، اختبار SRB، خلايا Vero، التعبير عن الكولاجين-1.

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Impact of an Educational Workshop on Improving Pharmacy Students' Knowledge About *Helicobacter pylori* Infection

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ABSTRACT

Aim: There is limited awareness about *H. pylori* in the community, and if improved, better disease control will be produced. This study aims to assess the level of knowledge among undergraduate pharmacy students regarding *H. pylori* diagnosis and management before and after delivering an educational pharmacy intervention.

Methods: This intervention study was initiated in May 2020, where 72 pharmacy students attended a workshop about *H. pylori* management and diagnosis. Students' knowledge about *H. pylori* infection was determined before and after the workshop. The intervention group attended a focused lecture about *H. pylori* combined with a case diagnosis and management simulation session. The control group self-reviewed a pamphlet related to the topic containing general information about *H. pylori*.

Results: Among the participants (n= 72), 58 (80.6%) had not attended a similar workshop previously. The intervention group demonstrated a significant improvement in their median knowledge score, rising from 9.0 (IQR: 8.0–11.0) pre-workshop to 11.0 (IQR: 10.0–12.0) post-workshop (P-value = 0.001). In contrast, the control group showed no significant change, maintaining a median score of 9.0 (IQR: 8.0–11.0) (P-value = 0.324). Additionally, while both groups had similar baseline knowledge scores, the intervention group achieved a significantly higher score after the workshop (P-value = 0.006).

Conclusion: The educational intervention resulted in a significant improvement in pharmacy students' knowledge about H. pylori. These findings underscore the importance of targeted educational activities in enhancing student preparedness for managing H. pylori cases in clinical practice.

Keywords: *H. pylori*; educational intervention; pharmacy student; knowledge; diagnosis.

1. INTRODUCTION

Helicobacter pylori (*H. pylori*) is recognized as a serious global health concern due to its high prevalence and significant epidemiological impact [1]. In Jordan, approximately 88% of the population tested positive for *H. pylori*, indicating a widespread issue [2]. This bacterium is associated with various diseases, including gastric cancer and other inflammatory conditions, underscoring the need for effective strategies to manage its prevalence and related health consequences [3, 4]. Notably, the Jordanian Ministry of Health has reported that gastric cancer ranks as the ninth most diagnosed cancer, comprising about 2.5% of all cancer cases in the country [5].

Several factors can increase the likelihood of *H. pylori* infection and compromise patient health [6, 7]. These include increased gastric acid production, tobacco smoke, and the use of certain pharmaceuticals, which may create

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an environment conducive to *H. pylori* colonization [6, 7]. While there is some association between dietary habits and chronic *H. pylori* infection [8], the role of micronutrient deficiencies remains less clear [9]. Moreover, contaminated water and inadequately sanitized food sources can harbor high concentrations of this bacterium, emphasizing the need for careful assessment of dietary intake [10].

There is a significant public lack of knowledge regarding *H. pylori*, highlighting the need for effective education and awareness campaigns [11, 12]. Pharmacists, as the most accessible healthcare providers, play a crucial role in educating the community about their medical conditions and the management of infections like *H. pylori* [13, 14]. As future pharmacists, students will be ideally placed to fill these knowledge gaps, utilizing the accessibility and affordability of pharmacy services to educate the community about medical conditions [15]. However, pharmacy students often have inadequate knowledge, leaving them with only a basic understanding of *H. pylori* [16].

Addressing this educational deficit is critical not only for enhancing the competencies of pharmacy students but also for ultimately reducing *H. pylori* infection rates in the community [17]. Given their accessibility, pharmacy students as a future pharmacists are ideally situated to educate the public on the risks associated with *H. pylori* and to promote health campaigns aimed at early detection and management [17, 18]. Continuous education for pharmacists and pharmacy students is essential [19-21]; equipping them with the necessary knowledge and skills for effective patient consultation, early referrals, and appropriate management strategies can significantly impact community health outcomes.

Implementing innovative educational strategies, such as targeted workshops and simulation-based learning, may enhance pharmacy students' understanding of *H. pylori* and improve their practical skills in managing related health issues. These initiatives are expected to foster a new generation of pharmacists who are better prepared to address healthcare challenges. In light of these considerations, this study aims to assess the effectiveness of specially designed educational workshops on pharmacy students' knowledge of *H. pylori* diagnosis and management. By evaluating the outcomes of these educational interventions, we hope to contribute valuable insights into improving pharmacy education and public health initiatives related to *H. pylori.*

2. METHODS

2.1 Study design, setting, and study subjects

This study adopted a pre-post interventional design that was conducted in the faculty of pharmacy at Applied Science Private University (ASU) in Amman-Jordan. Data collection took place in May 2020. During the study period, a convenience sample of pharmacy students from the third, fourth, and fifth year was invited to participate in this study to assess the impact of an educational workshop on their knowledge about *H. pylori* infection management and diagnosis. Students were randomized into two groups. A control group representing the students who will receive minimal education about *H. pylori* similar to what they actually receive in the curriculum, by self-reviewing a previously prepared pamphlet containing only general definitions or limited details about diagnosis and treatment. On the other hand, an intervention group was assigned to receive education for *H. pylori* on a different level. The type of education model followed here was not ordinary. Students had a hybrid experience between direct information exchange, the latest updates in diagnosis technologies, and patient case simulation trials to immerse the students' minds and passion into the education session without disconnecting out of boredom or lack of interest and also to form an impression on how to deal with *H. pylori* cases upon practice.

2.2 Sample size calculation

The sample size was calculated based on the results of a previous study that evaluated the impact of a learning

program in improving nurses' skills in the identification and classification of peptic ulcer disease cases [22]. In that study, the pooled standard deviation for the total skill scores for both the intervention and the control group was 1.51. Setting alpha at 0.05, the power of 80%, and using the following equation [23]:

$N= 2\ \sigma^2\ (Z_{Critical} + Z_{power})^2 /D^2$

Where,

σ is the pooled standard deviation for both groups.

Z critical value is equal to 1.96 for the 0.05 significance level.

Z power value is equal to 0.842 for the 80% statistical power.

D is the minimum expected difference between the two means which was set as 1.

Based on the above equation, the minimum required sample size to obtain a significant difference was calculated as 36 subjects per group.

2.3 Ethical considerations

The study protocol was approved by the Ethics Committee at the ASU (Approval number 2020-PHA-10). The study was conducted following the ethical standards outlined in the World Medical Association Declaration of Helsinki guideline [24]. Students were informed that their participation in the study is voluntary and that their responses will be kept confidential and analyzed only as part of a cohort. Written informed consent was obtained from all participants before the interview.

2.4 Questionnaire Development The study questionnaire was based on literature reviews [25-27], and underwent both content and face validation by three researchers with extensive expertise in this field. Content validation ensured that the questionnaire accurately covered all relevant topics related to *H. pylori* knowledge, while face validation confirmed that the questions were clear, relevant, and appropriate for the target population. The survey was divided into two main parts: (i) questions to determine participants' demographic characteristics, and (ii) questions to assess participants' general knowledge about *H. pylori*. For the knowledge questions, pharmacy students were awarded one point for correct answers and zero points for incorrect answers. Finally, a total knowledge scores out of 13 was calculated for each pharmacy student.

2.5 Data collection prior to the training workshop

Students of the third, fourth, and fifth years were invited to an educational workshop held at the faculty of pharmacy, aimed to increase their knowledge about disease signs, symptoms, and diagnosis methods. Online invitations were sent to students via social media websites in which participants had to state their names to be registered as a participant and be informed about the details of participation and the constituents of the workshop.

All 72 registered students were invited to participate in a one-hour simulated training workshop, they were asked to take their seats, where they found pre-workshop data collection forms placed on the front of each seat. The pre-workshop data collection form (required 15 minutes) consisted of 1) a consent form, where students read the objective of the study and then provide their signature as consent to participate in this study, and 2) a section about demographics and answered general questions about *H. pylori*, 3) also, students answered multiple close-ended questions to assess their general knowledge about *H. pylori* diagnosis and management.

Following the baseline data collection, participants were divided into two groups using a randomization table generated using the Social Science for Statistical Package software (SPSS) version 24 (SPSS Inc., Chicago, IL, USA) which resulted in 36 students assigned to the control group and 36 students assigned to the intervention group.

2.6 The education workshop

The control group students received an informative brochure to read about *H. pylori* epidemiology, signs, symptoms, and diagnosis methods, followed by a period for exchanging any questions that the students might have regarding the contents of the brochure. While the intervention group first received a 30-minute detailed

educational workshop that involve a lecture covering *H. pylori* epidemiology, resistance trends, complications, related comorbidities, old and current diagnostic technologies utilized in practice, and pharmacoeconomic assessment of different treatment regimens prescribed. Secondly, simulation training took part on how to distinguish and diagnose *H. pylori* infection which was presented to the students by the main researcher in this study and the simulator.

2.7 Data collection post the simulated training workshop

After the simulation training was completed, all students proceeded to complete the remaining parts of the survey, which required 15 minutes and included a second assessment of their knowledge about *H. pylori* diagnosis and management.

2.8 Statistical analysis

Data were analyzed using the statistical package for social science (SPSS) version 24 (SPSS Inc., Chicago, IL, USA). The descriptive analysis was done using median and interquartile range (IQR) for continuous variables and frequency (percentage) for categorical variables.

When comparing the difference between the two groups (control vs intervention), Mann Whitney U test was used for the continuous variable and chi-square/Fisher Exact tests for the categorical variables. To ascertain whether the educational intervention has an impact on students' knowledge about *H. pylori* diagnosis and management for both the control and intervention groups, the Wilcoxon signed-rank test was performed to assess the difference in the pre- and post-workshop knowledge score for each group. For statistical analysis, all tests were two-tailed and a P-value of less than 0.05 was considered statistically significant.

3. RESULTS

In this study, 72 pharmacy students volunteered to participate, of whom 57 (79.2%) were females and 15 (20.8%) were males. The median age of the students was 22 years (IQR: 21.0–23.0). Most participants were in their fourth year of study (n = 44, 61.1%). Table 1 presents the demographic characteristics of the study sample. When comparing the demographic characteristics of the control and intervention groups, no significant differences were found in age or gender (P > 0.05). However, a significantly higher proportion of students in the intervention group were in the fifth year (20, 55.6%) compared with the control group (4, 11.4%) (P < 0.001.

Table 1. Demographic characteristics of the study participants (n=72)

Parameter	Total study sample n= 72	Control group n= 36	Intervention group n= 36	P-value
Age (years) Median (IQR), [range]	22 (2.0), [20-29]	21 (1.0). [20-29]	22 (2.0), [20-27]	0.103
Gender, n (%) ○ Males ○ Females	15 (20.8) 57 (79.2)	8 (22.2) 28 (77.8)	7 (19.4) 29 (80.6)	0.772
Academic year, n (%) ○ Third year ○ Fourth year ○ Fifth year	4 (5.6) 44 (61.1) 24 (33.3)	0 (0.0) 32 (88.9) 4 (11.1)	4 (11.1) 12 (33.3) 20 (55.6)	<0.001*

Using Chi-square test for categorical variables, and Mann Whitney for continous variable, * significant at significance level of 0.05

In this study, most of the students have heard of *H. pylori* before taking the workshop (n= 66, 91.7%) and the remaining 8.3% (n= 6) of students have never heard of it before. The majority of participants haven't been involved in any similar workshops that discuss *H. pylori* infection (n=58, 80.6%) while the other 19.4% (n=14) took previous workshops about this topic (Figure 1).

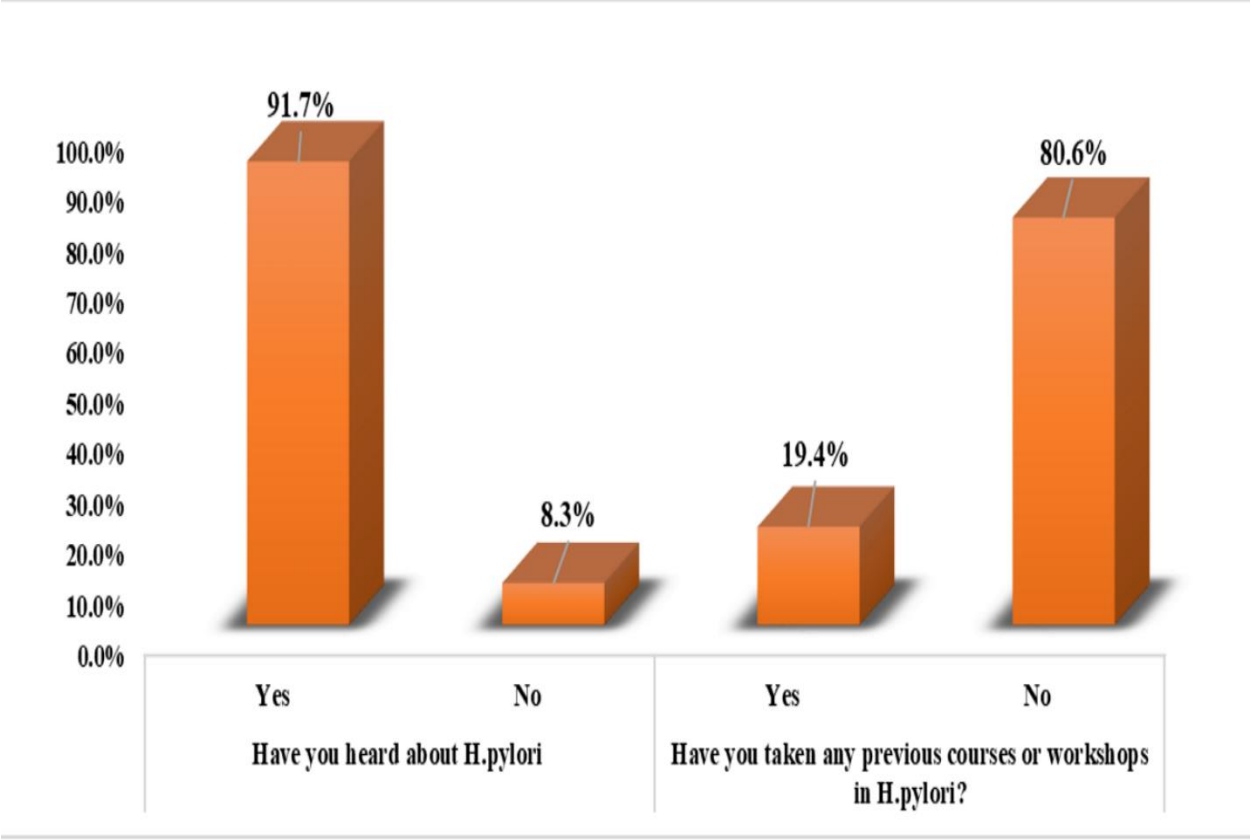

Figure 1. Students' awareness and prior educational exposure to *H. pylori* (n= 72)

After evaluating students' baseline knowledge about *H. pylori* (Table 2), almost half of the students (n=34, 47.2%) realized that the chance of *H. pylori* prevalence increases with time in our community. The majority of students knew that an infection of *H. pylori* can occur at any age (n=64, 88.9%). When asked if there are certain food types associated with getting an infection, 68.1% (n=49) answered correctly and 31.4% (n=23) didn't. When asked about the relationship between *H. pylori* on one hand and good hygienic practice and socioeconomic status on the other hand, 79.2% (n=57) answered correctly. The students were almost equally divided in their answers when asked if *H. pylori* can be contracted via different routes with 48.6% (n=35) agreeing with this statement, and 51.4% (n=37) disagreeing, incorrectly.

Table 2. Assessment of student's baseline knowledge about *H. pylori* (n= 72)

Statement	Correct answer	Number (%) of participants with correct answers			
		Total study sample n= 72	Control group n= 36	Intervention group n= 36	P-value#
Prevalence of *H. pylori* is increasing with time in Jordan	True	34 (47.2)	17 (47.2)	17 (47.2)	1.000
Infection with *H. pylori* can occur at any age	True	64 (88.9)	32 (88.9)	32 (88.9)	1.000
There are certain food types associated with *H. pylori* infection	True	49 (68.1)	24 (66.7)	25 (69.4)	0.800
Good hygiene practice reduces *H. pylori* infection rates	True	57 (79.2)	24 (66.7)	33 (91.7)	0.009*
Socioeconomic status has no association with *H. pylori*	False	57 (79.2)	26 (72.2)	31 (86.1)	0.147
There are different routes of infection for *H. pylori*	True	35 (48.6)	14 (38.9)	21 (58.3)	0.099
There is only one treatment regimen for *H. pylori* infection according to the guidelines	False	56 (77.8)	29 (80.6)	27 (75)	0.571
Therapy plan can last for more than 14 days	True	46 (63.9)	24 (66.7)	22 (61.1)	0.624
Symptoms of infection can include nausea, mucous in stool, reduced appetite, unintentional weight loss	True	66 (91.7)	33 (91.7)	33 (91.7)	1.000
Physicians usually depend on reported symptoms only to prescribe the proper medication	False	46 (63.9)	26 (72.2)	20 (55.6)	0.141
Once the patient is diagnosed and treated, the infection cannot reoccur	False	52 (72.2)	31 (86.1)	21 (58.3)	0.009*
Diagnostic lab tests for *H. pylori* only use stool-based samples	False	44 (61.1)	22 (61.1)	22 (61.1)	1.000
Primary lab tests can be confirmed by other blood-based (serum) tests	True	51 (70.8)	25 (69.4)	26 (72.2)	0.795

Using Chi-square test, * significant at significance level of 0.05

Fifty-six of the students (77.8%) knew that there is more than one treatment regimen for *H. pylori*, and 64.0% (n=46) knew that the treatment regimen can last for more than 14 days. The vast majority of the students recognized some of the possible symptoms of *H. pylori* (n=66, 91.7%). Twenty-six (36.1%) students believed that physicians only depend on reported symptoms from patients to diagnose them, whereas 46 students (63.9%) believed the opposite.

Over two-thirds of the participants knew that once a patient is treated, the infection cannot reoccur and that primary laboratory tests can be confirmed by blood-based (serum) tests (n = 52, 72.2%; n = 51, 70.8%, respectively). Moreover, a substantial proportion of students knew that samples other than stool can be used for screening (n = 44,

61.1%), as shown in Table 2.

Following the educational workshop, the intervention group demonstrated a significant improvement in their understanding of both the prevalence of H. pylori and treatment regimens. Specifically, the proportion of students who acknowledged that the prevalence of H. pylori is increasing over time in Jordan rose from 17 (47.2%) pre-workshop to 32 (88.9%) post-workshop (p-value <0.001). Additionally, students improved their knowledge regarding treatment options, with those recognizing that multiple treatment regimens are available increasing from 27 (75%) to 34 (94.4%) (p-value = 0.039). These results are summarized in Table 3.

Table 3. Comparison between students' correct answers pre-workshop and post-workshop for the intervention group, (n= 36)

Statement	Correct answer	Number (%) of participants with correct answers		P value
		Pre-workshop	Post-workshop	
Prevalence of *H. pylori* is increasing with time in Jordan	True	17 (47.2)	32 (88.9)	<0.001*
Infection with *H. pylori* can occur at any age	True	32 (88.9)	31 (86.1)	1.000
There are certain food types associated with *H.pylori* infection	True	25 (69.4)	31 (86.1)	0.146
Good hygiene practice reduces *H. pylori* infection rates	True	33 (91.7)	36 (100)	NA
Socioeconomic status has no association with *H. pylori*	False	31 (86.1)	26 (72.2)	0.227
There are different routes of infection for *H. pylori*	True	21 (58.3)	30 (83.3)	0.120
There is only one treatment regimen for *H.pylori* infection according to the guidelines	False	27 (75)	34 (94.4)	0.039*
Therapy plan can last for more than 14 days	True	22 (61.1)	22 (61.1)	1.000
Symptoms of infection can include nausea, mucous in stool, reduced appetite, unintentional weight loss	True	33 (91.7)	35 (97.2)	0.625
Physicians usually depend on reported symptoms only to prescribe the proper medication	False	20 (55.6)	24 (66.7)	0.388
Once the patient is diagnosed and treated, the infection cannot reoccur	False	21 (58.3)	23 (63.9)	0.727
Diagnostic lab tests for *H.pylori* only uses stool based samples	False	22 (61.1)	25 (69.4)	0.549
Primary lab tests can be confirmed by other blood based (serum) tests	True	26 (72.2)	27 (75.0)	1.000

* Significant at significance level of 0.05, # using McNemar test, N/A- Not Applicable

When comparing baseline knowledge between the intervention and control groups (Table 4), no significant differences were observed in 11 out of the 13 knowledge statements (P > 0.05). However, a significantly higher proportion of students in the intervention group initially knew that good hygiene practices reduce *H. pylori* infection rates compared with the control group (33, 91.7% vs. 24, 66.7%, respectively; P = 0.009). Conversely, a higher proportion of students in the control group initially recognized that the infection can reoccur even if the patient was previously treated compared with the intervention group (31, 86.1% vs. 21, 58.3%, respectively; P = 0.009).

Table 4. Comparison of the intervention and control groups, concerning their knowledge score, pre and post the intervention

Parameter		Control (n= 36)	Intervention (n= 36)	P-value$
		Median (IQR)	Median (IQR)	
Knowledge score (medina (IQR))	Pre-intervention	9.0 (3.0)	9.0 (3.0)	1.000
	Post-intervention	9.0 (3.0)	11.0 (2.0)	0.006*
P-value#		0.324	0.001	

Using Wilcoxon sign rank test, $ Using Mann Whitney U test, * significant at significance level of 0.05

When comparing the overall knowledge score before and after the workshop for both the control and intervention groups (Table 4), students in the intervention group showed a significant improvement in their median knowledge score from 9.0 (IQR: 8.0–11.0) pre-workshop to 11.0 (IQR: 10.0–12.0) post-workshop (P-value= 0.001). However, there was no significant improvement in the knowledge score of the control group, which had a pre-workshop median knowledge score of 9.0 (IQR: 8.0–11.0) and a post-workshop median score of 9.0 (IQR: 8.0–11.0) (P-value=0.324). When comparing the two groups, there was no significant difference in the median knowledge score prior to the intervention (median = 9.0, IQR: 8.0–11.0 for both groups (P-value= 1.000), while the intervention group showed higher median knowledge score following the workshop compared to the control group (11.0 (IQR: 10.0–12.0) vs 9.0 (IQR: 8.0–11.0), P-value = 0.006).

4. DISCUSSION

There are many studies that addressed the topic of *H. pylori* in areas that focused on new ways to confirm a positive diagnosis or looked deeply into trends of disease spread and prevalence among smaller communities and all the way to a worldwide level[28-30]. However, to our knowledge, this is considered to be one of the first studies assessing undergraduate pharmacy students' knowledge regarding *H. pylori* management and diagnosis specifically before and after receiving an educational workshop.

Putting out more qualified and up-to-par pharmacy students as part of the healthcare provider's team is a goal that every entity in the world aims to achieve. Achieving this goal means better health services for everyone, and this can only be done through the continuous development of educational programs and the ingenuity of ways to deliver knowledge and information to students.

In this study we took the topic of *H. pylori* management and diagnosis; we attempted to utilize it as a model for similar topics to be covered within the curriculum and designed different educational scenarios to assess the impact of such activity and how much difference it can make in the quality of the produced educational outcome for pharmacy students.

Although a small percentage of the students reported

having never heard of this topic before, the majority haven't been involved in any similar workshops that discuss *H. pylori* infection. It is noteworthy that around 58% of participants were knowledgeable about the causative factors of *H. pylori* infection. This percentage is higher than the results of previous study targeting healthcare providers, where only 41.5% of healthcare providers demonstrated knowledge in this area [31]. Student's awareness about food involvement as a consumed carrier for infection was good, as 68% of them were certain about the role of certain food types in association with *H. pylori* infection, which is confirmed in a related study [32]. [31][30][29]Most of the participants knew that *H. pylori* infection can occur at any age (89%) and that it increases with time which supports the results of Hussen et.al [33]. Students in intervention and control groups (72.2%) had realized the association between the chance of developing an infection with *H. pylori* and the socioeconomic status of individuals, which reaffirms the findings of similar studies [34, 35]..

The educational workshop for the intervention group significantly improved knowledge about *H. pylori* management and diagnosis, as evidenced by the increase in overall knowledge scores compared to the control group. This highlights the importance and apparent impact of such activities outside of the traditional educational setting similar to what was reported by several previous studies [36, 37]. The results of this research amplify the importance and need for specially designed, more focused, and reality-simulating educational activities like workshops or other ventures to be utilized and implicated within the high number of courses that students take before graduating.

The results of this study support the proposed hypothesis that implementing well-designed pharmacy education programs on *H. pylori* infection management and diagnosis can positively impact pharmacy students' knowledge. Expanding such educational activities to a broader level and integrating them into multiple courses within the students' curriculum could significantly enhance their ability to address similar issues when they begin professional practice.

Another notable benefit of these programs is their capacity to cover topics that may not have been thoroughly addressed or formally planned by faculty due to time constraints, limited resources, or dense curricula. Such initiatives provide both students and educators with opportunities to broaden their knowledge base, foster creativity, and promote innovative thinking in educational environments where the primary focus is on learning rather than grades.

This study has several limitations that should be acknowledged. First, although participants were randomly assigned to the control and intervention groups, a significant difference in academic year levels was observed between the two groups ($P < 0.001$). This difference may affect the comparability of results, as students in different academic years may have varying levels of prior knowledge and experience related to *H. pylori* management and diagnosis. Also, differences in academic level may still influence students' ability to absorb and apply new information. Future studies should aim to recruit participants from similar academic levels to ensure more valid comparisons. Also, we did not collect detailed demographic information, particularly GPA scores, for both the control and intervention groups, which prevents us from assessing the potential influence of academic performance on the outcomes of the study. Additionally, the impact of the educational intervention was assessed immediately after the workshop, which does not account for long-term retention of knowledge and its application in practice. Longitudinal studies are needed to evaluate the sustainability of the knowledge gained over time. Finally, the sample was drawn from a single faculty, which may limit the generalizability of the findings. Including a more diverse range of participants from multiple faculties could provide a more comprehensive

understanding of pharmacy students' knowledge of *H. pylori*.

Also, future research involving specially designed educational activities needs to be promoted more to extract ideas, innovations, and ways to improve educational experiences and increase our understanding of the current quality of delivered educational programs and what needs to be improved depending on priorities and in a way that goes hand in hand with official regulatory bodies responsible for education on a country level.

In conclusion, this study demonstrates that targeted educational workshops significantly enhance pharmacy students' knowledge of *H. pylori* management and diagnosis. The marked improvement in knowledge scores among the intervention group highlights the effectiveness of incorporating critical health topics into pharmacy curricula. Given the high percentage of students previously unfamiliar with *H. pylori*, the findings highlight the importance of ongoing educational development to prepare future pharmacists for real-world challenges. Future research should focus on the long-term impacts of such interventions to ensure knowledge retention and practical application.

Conflict of interest

The Authors declare that there is no conflict of interest.

Disclosure statement(s)

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أثر ورشة العمل التعليمية على تحسين معرفة طلبة الصيدلة بعدوى بكتيريا الملوية البوابية

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ملخص

الهدف: تُعد المعرفة المحدودة بعدوى بكتيريا الملوية البوابية في المجتمع عاملاً مساهماً في قلة السيطرة على المرض. تهدف هذه الدراسة إلى تقييم مستوى المعرفة لدى طلبة مرحلة البكالوريوس في الصيدلة فيما يخص تشخيص وعلاج عدوى الملوية البوابية، قبل وبعد تنفيذ تدخّل تعليمي صيدلاني.

المنهجية: أُجريت هذه الدراسة التدخلية في شهر أيار/مايو من عام 2020، حيث شارك 72 طالبًا من كلية الصيدلة في ورشة عمل تتعلق بتشخيص وعلاج عدوى الملوية البوابية. تم تقييم معرفة الطلبة حول هذه العدوى قبل وبعد الورشة. تلقى أفراد مجموعة التدخّل محاضرة مركزة عن الملوية البوابية، بالإضافة إلى جلسة محاكاة حول تشخيص الحالة وإدارتها. بينما قامت المجموعة الضابطة بمراجعة كُتيّب يحتوي على معلومات عامة عن الملوية البوابية.

النتائج: بين المشاركين(n = 72) ، أشار 58 منهم (80.6%) إلى أنهم لم يسبق لهم حضور ورشة عمل مماثلة. أظهرت مجموعة التدخّل تحسنًا ملحوظًا في متوسط درجات المعرفة، حيث ارتفع من 9.0 (النطاق الربيعي: 8.0–11.0) قبل الورشة إلى 11.0 (النطاق الربيعي: 10.0–12.0) بعد الورشة (قيمة P = 0.001). في المقابل، لم تُظهر المجموعة الضابطة تغييرًا يُذكر، إذ بقي متوسط الدرجة 9.0 (النطاق الربيعي: 8.0–11.0) قيمة (P = 0.324)، كما أن كلا المجموعتين كانتا تمتلكان مستويات معرفة متقاربة في البداية، إلا أن مجموعة التدخّل سجلت درجات أعلى بشكل ملحوظ بعد الورشة قيمة.(P = 0.006

الاستنتاج: أدّى التدخّل التعليمي إلى تحسّن كبير في معرفة طلبة الصيدلة بعدوى بكتيريا الملوية البوابية. وتُبرز هذه النتائج أهمية الأنشطة التعليمية الموجهة في تعزيز جاهزية الطلبة للتعامل مع حالات عدوى الملوية البوابية ضمن الممارسة السريرية.

الكلمات الدالة: بكتيريا الملوية البوابية؛ تدخّل تعليمي؛ طلبة الصيدلة؛ المعرفة؛ التشخيص.

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Caraway Seed Extract and Lactation: *in vivo* study

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ABSTRACT

The aim of this study is to investigate the effect of caraway seed aqueous extract on milk production in female rats and elucidate its bioactive components. We used three groups of six virgin rats and three groups of six lactating rats. We kept each mother rat and her six pups apart. From the third to the seventeenth day of breastfeeding, these groups of animals received distilled water, metoclopramide (5 mg/kg), and aqueous extracts of caraway seed (200 mg/kg) daily by mouth. We used the indirect method, which established a link between the amount of milk nursing rats produced and the weight gain of pups who received no food. We also measure the prolactin levels of female rats who have never been pregnant on specific days. On Day 14, the groups that received caraway seed fluid extracts (200 mg/kg) or metoclopramide (5 mg/kg) showed significantly higher prolactin levels ($P > 0.01$ and $P > 0.001$, respectively). Studies have shown that caraway seed significantly increases prolactin levels ($P < 0.05$) as early as Day 7. Only with (5 mg/kg) of metoclopramide did milk production and pup weight gain go up significantly ($P > 0.05$). An extract of caraway seed affects prolactin levels in rats, but it does not affect milk production. Further research is necessary to explore the potential therapeutic benefits of caraway seed.

Keywords: Galagtagouge, *Carum carvi*, Metoclopramide, Milk production, Prolactin, Medicinal plants.

1. INTRODUCTION

Breastfeeding babies gives them the best diet, boosts their immune systems, and has many other health benefits that last a lifetime for both mother and child. One major issue that has dominated the lactation field for many years is hypogalactia (reduced milk production).[1] It is the most frequent cause of breastfeeding failure, leading to the cessation of breastfeeding. [2] According to reports, >20% of mothers who breastfed their infants experienced postpartum hypogalactia. The number of women with hypogalactia has gone up because the average age of mothers is getting older and the number of cesarean sections is going up all over the world.[3] 92% of children in Jordan experience breastfeeding at some point, making it a country with a high breastfeeding prevalence.[4] Nonetheless, prior research conducted in Jordan has indicated negative sentiments on breastfeeding practices. Furthermore, Jordanian population and family health surveys show that the percentage of six-month-old infants exclusively nursing is below optimal and has been falling, from 40% in 2007 to 26% in 2018.[4]

Medicinal plants are still used for medical purposes, especially in developing nations where access to contemporary therapeutic systems may be limited. [5]Asadbeigi et al. (2014) say that ethnomedicines are in high demand because they are a cheap, easy-to-find, and safe source of active compounds that can be used in pharmaceuticals.[6] Nowadays, medicinal plants continue to play an important role in the treatment of a variety of illnesses, particularly in rural areas due to budgetary constraints and inconvenience, as well as health concerns.[7] We can use herbal and pharmaceutical galactagogues to treat women who don't

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make enough milk and don't respond to lactation counseling, as well as adoptive parents who want to start breastfeeding. Though the market for pharmaceutical and herbal galactagogues is growing, there is little information on how to use them.[1]

Caraway (*Carum carvi* L.), belongs to the family Umbelliferae. It is one of the first cultivated herbs in Asia, Africa, and Europe. Folk medicine particularly uses the plant's dried fruits and leaves to treat digestive disorders such as stomach aches, constipation, flatulence, and nausea. These oldest herbs are filled with a specific, pleasant aroma. Its aromatic properties make it a valuable flavoring agent. [8] Moreover, liqueurs, mouthwashes, toothpastes, perfumes, soaps, and cosmetics use it. [9] Traditional Persian medicine uses caraway, a galactogogue, to reduce excessive breast milk production. [10,11]

Up to our knowledge, there is not enough data about the exact galactogogue effect of caraway seed aqueous extract. The current study aimed to evaluate the effect of caraway aqueous extract on milk production in rats and identify the phytoconstituents in caraway seed aqueous extract.

MATERIAL AND METHODS

Plant materials

We conducted research on caraway seeds. In 2022, we procured caraway seeds from the traditional herbal market in Zarqa, Jordan. Associate Professor Mohammad M. Al-Gharaibeha from the Faculty of Agriculture, Jordan Department of Plant Production, University of Science and Technology assisted in taxonomically identifying the plant through direct comparison with authenticated samples. Zarqa University's College of Pharmacy (Pharmacognosy and Phytochemistry Lab) received the voucher specimen (No. CAR-2022).

Preparation of caraway extract

Experimental animals

Female Wistar laboratory rats (weight ranges of 170-200 g) were housed in cages with controlled temperatures (22-25°C) and relative humidity (50-60%). They provided unlimited access to normal pellet food and water. All animal care and use procedures followed established ethical criteria, and all experimental protocols were approved by the Research and Ethics Committee at the Faculty of Pharmacy-Applied Science University in Amman, Jordan (2022-PHA-23). Eighteen female Wistar rats weighing 170-200 g were mated with male rats a few days before parturition at a regulated temperature (22-25 °C) and relative humidity (50-60%). Each pregnant female Wistar rat was segregated from the other pregnant female Wistar rats. The rats were allowed to give birth to their offspring. Each mother was separated from her pups immediately after parturition. The number of pups per breastfeeding rat was six, and parturition occurred on Day 1 of lactation.[13]

Experiment design

Lactating rats were sorted into three groups (n= 6) using the procedure outlined.[13] Each group contains six lactating rats. From Day 3 to Day 17, we administered the following treatments to the lactating rats:

- **Group I**: Served as a negative control and was given unlimited access to water.
- **Group II**: Metoclopramide (5mg/kg) was used as the standard treatment.
- **Group III**: Caraway seed aqueous extract (200 mg/kg) was used.

Metoclopramide and plant extracts are dissolved in distilled water. The prepared solution was given to the rats through oral gavage. Based on prior research [14,15] the dose of metoclopramide (5 mg/kg) was chosen.

The puppies were not given any treatment, but their weights were taken at various times of the day from Day 3 to Day 17.

Milk production and body weight measurements

The weights of the puppies (P1) were measured at 8:00 AM subsequent to a period spent overnight with their maternal figures. They were subsequently segregated from the nursing rats for a duration of four hours. At 12:00 PM, a second weighing (P2) was conducted, after which they were reintegrated with the nursing rats for one hour of

suckling. The third weight (P3) was ascertained at 1:00 PM to evaluate the quantity of milk ingested by the puppies. The determination of milk yield is predicated upon the disparity in weight between the puppies before and after the feeding sessions. This yield is computed through the utilization of a weight loss correlation coefficient. The absorbed milk by the puppies serves to furnish the requisite energy for their physiological activities such as locomotion and respiration. The formula employed to accurately predict milk yield is articulated as follows:[16]

Milk yield(g)=(P3-P2)+[(P2-P1)/4]

- (P3-P2) represents the growth in puppy weight subsequent to lactation,
- (P2-P1)/4 denotes the weight loss correlation coefficient.

Prolactin levels in blood measurements

Eighteen virgin rats weighing (170-200 g) were separated into three groups of six animals each for fourteen days.

- **Group I**: Served as a negative control and was given unlimited access to water.
- **Group II**: Metoclopramide (5 mg/kg) was used as the standard treatment.
- **Group III**: Caraway seed aqueous extract (200mg/kg) was used.

Virgin rats were given a single dose of caraway aqueous extract and metoclopramide every day. The serum prolactin level was tested on Days 0, 7, and 14. Blood samples were drawn from the animals' retro-orbital plexus and centrifuged at 1000 rpm for 20 minutes.[17] After collecting the supernatant, the serum prolactin level was determined using an immunoenzymatic technique (ELISA Kit).[18]

Caraway seed aqueous extract bioactive compound identification

UPLC equipped with Q-TOF/MS was used to characterize bioactive chemicals. The UPLC chromatographic conditions are shown in (Table 1).

Table 1: Summary of chromatographic conditions

UPLC conditions	**Injection volume**	**Autosampler temperature**	**Column oven temperature**	**Total run time**
	3µl	8ºC	40°C	35min.
Chromatography	**Mobile phase**	Solvents: (A) Water with 0.05% formic acid (B) Acetonitrile Gradient: 0 – 27 min linear gradient from 5% - 80% B; 27 – 29 min 95% B; 29.1 min 5% B		
	Column type	Bruker Daltonik C-18 column (100× 2.1mm× 1.8µm)(120 Aº)		

Ion Source's Apollo II ion funnel electrospray source was employed as a source in both positive and negative modes. The following were the mass spectrometry conditions:

- The capillary voltage was 2500 volts (VT).
- The nebulizer gas pressure was 2.0 bar.
- The flow rate of dry gas (nitrogen) was 8 L/min.
- The dry temperature was 200 degrees Celsius.

The mass resolution was 50000 FSR (Full Sensitivity Resolution), and the mass accuracy was 1 ppm. The TOF repetition rate was up to 20 kHz. Data analysis 4.2 software was used to analyze the MS data (Bruker Daltonics, Bremen, Germany).

Statistical analysis

Using IBM SPSS software, a one-way ANOVA followed by *Dunnett's post-hoc* test was performed on all examined parameters (Version 22.0). The information was reported as mean ± SD. P values of less than 0.05 were deemed statistically significant. P values less than 0.01 were deemed statistically significant. P values of less than 0.001 were considered statistically significant.

The effect of caraway seed aqueous extract on milk production

The female rats' milk production was monitored daily before and after the test drugs were administered. The data revealed that the milk yield of each experimental group's rats was not proportional to the number of days of lactation (Figure 1(A)).

When compared to the negative control group, caraway seed aqueous extract (200 mg/kg) in the treated groups did not substantially improve milk production ($P > 0.05$). Metoclopramide (5 mg/kg) significantly enhanced milk production in nursing rats ($P > 0.05$) (Figure 1(B)).

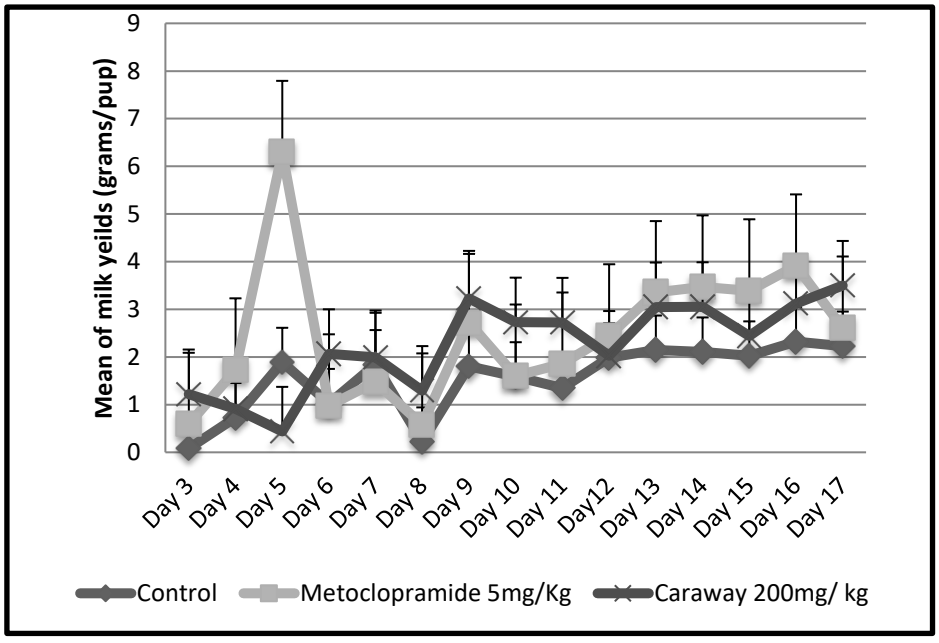

Figure 1 (A): Effect of the aqueous seed extract of caraway on the daily milk production in female Wistar rats from Day3 to Day17 of lactation. The values as expressed mean ± SD, with n=6.

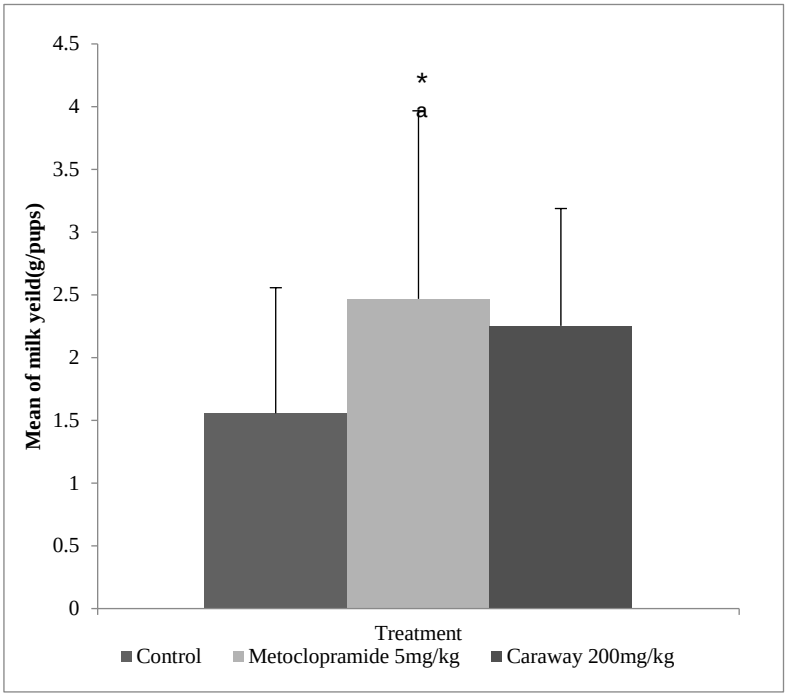

Figure 1(B): Effect of the aqueous seed extract of caraway on the average milk quantity in female Wistar rats from Day 3 to Day17 of lactation. Mean ± S.D (n = 6), *P<0.05, a: Compared with control.

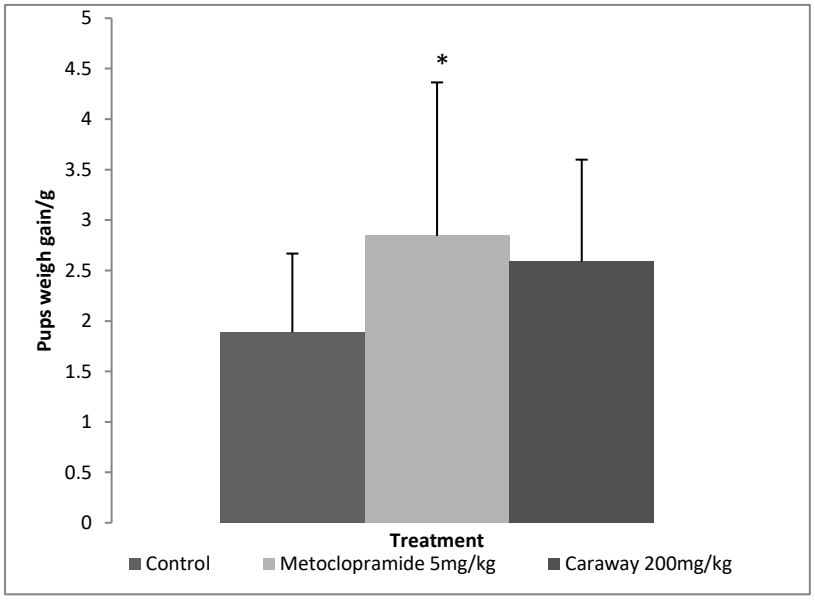

Figure 2: Effect of the aqueous seed extract of caraway on the average pups weigh gain from Day 3 to Day17 of lactation. Mean ± S.D (n = 6), *P<0.05, a: Compared with control.

Effect of the aqueous extract of caraway seed on pups' weight gain

Metoclopramide (5 mg/kg) showed significant increases ($P< 0.05$) in weight gain in lactating rats. In contrast, caraway seed aqueous extract (200 mg/kg) did not significantly increase the weight gain of pups ($P > 0.05$) (Figure 2).

Evaluation of serum prolactin levels

Metoclopramide (5 mg/kg) and caraway seed extract (200 mg/kg) were given to virgin female Wistar rats for 14 days. On Days 0, 7, and 14, prolactin levels were measured. No treatment resulted in prolactin levels higher than (0.9 ng/ml) on Day 0. On Day 7, caraway seed extract enhanced prolactin secretion considerably ($P <0.05$).

On Day 14, caraway (200 mg/kg) and metoclopramide (5 mg/kg) showed significantly increased prolactin levels ($P< 0.01$, $P<0.001$).

Identification of bioactive compounds in aqueous seed extract of caraway

The results showed the identification of many bioactive compounds in caraway seed aqueous extract, as well as a complete list of identified compounds together with their phytochemical class and chemical formula (Table 3).

Table 2: Effect of the aqueous seed extract of caraway on the serum prolactin level in virgin female rat.

Prolactin concentrations induced by treatments (ng/ml)			
Study period	**Control**	**Metoclopramide (5mg/kg)**	**Caraway aqueous extract (200mg/kg)**
Day 0	<0.9	<0.9	<0.9
Day 7	<0.9	0.98 ± 0.35[a]	1.56 ±0.68*[a]
Day 14	<0.9	6.9 ± 1.40***[a]	3.2 ± 0.96**[a]

**P<0.01, ***P<0.001. a: Compared with control group

Table 3: The identified phytoconstituents and their phytochemical class in the aqueous extract of caraway seed.

N	RT (min)	Identified compound name	Phytochemical class	Chemical formula	Ion mode
1.	0.59	Starch	Polysaccharide	$C_6H_{12}O_6$	[M-H]-
2.	0.59	Nicotinic acid	Pyridinecarboxylic acids.	$C_6H_5NO_2$	[MH]+
3.	0.64	Sugars	Carbohydrate	$C_{12}H_{22}O_{11}$	[MH]+
4.	1	Succinic acid	Dicarboxylic acid	$C_4H_6O_4$	[M-H]-
5.	1.45	Octadecanoic acid	Poly unsaturated fatty acid	$C_4H_6O_4$	[M-H]-
6.	5.64	Hyperoside	Flavanol glycoside	$C_{21}H_{20}O_{12}$	[MH]+
7.	6.75	Kaempferol	Tetrahydroxyflavone	$C_{15}H_{10}O_6$	[MH]+
8.	6.92	4 or 7 Hydroxy Coumarin	Coumarin derivative	$C_9H_6O_3$	[MH]+
9.	20.39	Caffeic acid	Phenolic acid (Cinnamic acid derivatives)	$C_9H_8O_4$	[MH]+
10.	24.05	18-Beta-glycyrrhetinic acid	Triterpenoid	$C_{30}H_{46}O_4$	[MH]+
11.	24.33	Tetradecanoic acid	Saturated fatty acid	$C_{14}H_{18}O_2$	[M-H]-

Compounds listed in order of retention time; RT= retention time (as min.)

DISCUSSION

The amount of milk each group of rats made was not related to the number of days they were lactating. This result is the same as what other studies have found about *Musa x paradisiaca* (Musaceae), *Euphorbia hirta* (Euphorbiaceae), *Pimpinella anisum* (Umbelliferae), and *Nigella sativa* (Ranunculaceae) as galactogogues.[19, 20, 21,17] The fact that mammary cells grow and die in the mammary glands at different times during lactation shows that milk production and days of lactation are not directly related. Because of this, the speed at which milk is made changes based on the phase of lactation. [22]

On Days 0, 7, and 14, rats that had never become pregnant before were given blood prolactin tests. To find out how galactagogues work, this was done.

The findings indicate that none of the treatment groups were successful in provoking a heightened prolactin response in virgin female rats at the onset of the experiment. This observation aligns with prior research findings. [17, 13]

Furthermore, it was observed that administration of aqueous extract of caraway seeds at a dosage of (200 mg/kg) led to a modest increase in lactation; however, this augmentation was not statistically significant. Hamed et al. (2015) have posited that the weight gain exhibited by offspring serves as an indicator of maternal lactation capability.[23]Contrary to expectations, supplementation of pups with caraway seed extract at the aforesaid dosage did not increase their weight gain ($P > 0.05$). This absence of accelerated weight gain suggests a potential correlation between insufficient milk production and the observed lack of weight gain in offspring.

Conversely, it was noted that the aqueous extract of caraway seeds, also administered at a dosage of (200 mg/kg), caused a notable increase in prolactin levels as early as Day 7, specifically in terms of serum prolactin release.

The effects of caraway seeds on milk production and prolactin release appear to be depending upon various factors such as parity, maternal nutrition, and hydration status. For instance, nutritional deprivation in offspring may result in increased milk consumption relative to satiated counterparts during lactation. [24]

During the lactation period of rat pups, significant developmental milestones occur. Primarily, a apparent modulation of sucking behavior emerges by Day 10 postpartum, indicative of a natural regulatory mechanism. Subsequently, by Day 14, observable alterations obvious in the maternal response to pup-initiated stimuli, notably in reaction to a nip or milk release. [25]

Moreover, the efficacy of nipple attachment necessitates sensitivity to a confluence of internal and external variables, including the pup's level of deprivation and the olfactory cues emanating from the lactating ventral surface. [26] Vorherr et al. (1967), Lincoln et al. (1973), and Drewett et al. (1974) have extensively explored facets of this phenomenon. Drewett et al. (1974), for instance, studied the constituent elements of rat pup behavior, delineating "treading" and "stretching" as salient components. [27,28,29]

Clearly, rat pups commonly disengage from the nipple following milk release, notwithstanding satiety, displaying a proclivity for locomotion to access alternative sources. The impetus driving this behavior remains speculative, although a plausible hypothesis implicates olfactory cues, potentially mediated by hormonal responses to oxytocin in the mammary gland region.[30]

Furthermore, it is noteworthy that each milk ejection event furnishes the pup with pertinent information regarding the occurrence and adequacy of milk uptake from the respective nipple. Consequently, in instances where milk is scant or absent despite a milk ejection event, the pup's instinctual response to seek an alternative nipple for sustenance appears rational and adaptive.

Also, the number of cells that make milk in the mammary glands has a big effect on milk production. Three-quarters of the increase in rats' daily milk production between birth and peak lactation can be explained by cell growth during early lactation. The other

quarter can be explained by an increase in the activity of cells that were already there. [31]

Overall, both estrogen and sucking cause prolactin to be made by female rats. This is done by blocking the dopaminergic tone in the hypothalamus and stimulating it with a prolactin-releasing hormone, probably oxytocin. [32]

Now, let's talk about caraway seeds. A review of the studies shows that there is not much known about how caraway affects lactation. In this study, caraway seeds had the same effect on prolactin levels as an earlier study, which found that prolactin levels went up. [33]But it doesn't seem to match up with milk production, which might have something to do with the other things we've talked about. In another study, five nursing women were given no herb for 5 days and then 15 mL of a 10% infusion of caraway seeds three times a day for 10 days. Every day, the amount of milk was measured, and the amount of fat in the milk was tested on days 5, 10, 15, and 20. There was no change in the amount of milk or how much fat it had. [34]Ethanol or aqueous caraway extracts, on the other hand, had estrogenic effects at doses higher than (200 mg/kg). [35]

Additionally, LC-MS/MS analysis of caraway aqueous extracts showed the presence of polyphenolic compounds like hyperoside, kaempferol, vitexin, etc. These bioactive compounds act as antioxidants; [37]a study showed that the antioxidant compounds increase the prolactin hormone by affecting the hypothalamic-pituitary axis. [38] Thus, that might be interpreted as a significant effect of caraway seed aqueous extracts on prolactin levels in this study.

In the current study, metoclopramide served as a standard medication. Metoclopramide is considered a safe and effective stimulant of prolactin release. It achieves this by blocking dopamine receptors in the hypothalamus and reducing prolactin inhibitory factor. [39]Metoclopramide (5 mg/kg) significantly increased milk production and serum prolactin levels in this study.

CONCLUSION

An aqueous caraway seed extract was found to have a statistically significant impact on prolactin levels in the blood of nulliparous rats. This impact may be due to the bioactive chemicals included in the extract. Further work is needed to clarify the underlying process that causes a difference between prolactin levels and milk production. These findings add to the increasing evidence that medicinal plants have the potential to be used as new therapeutic agents in current medical practice.

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Ethical Approval

The study protocol was approved by approved by the Research and Ethics Committee at the Faculty of Pharmacy-Applied Science University in Amman, Jordan (2022-PHA-23).

Disclosure

The authors report no conflicts of interest in this work.

Abbreviations

ELISA, enzyme-linked immune-sorbent assay; FSR, full sensitivity resolution; Kg, kilogram; kHz, Kilohertz; L, Litter; LC-MS, Liquid-chromatography–mass spectrometry; Mg, Milligram; µg, Microgram; µL, Microlitre; ML, Millilitte Min, Minutes; Q-TOF,Quadrupole-Time-of-flight;.RT, Retention time; SD, Standard Deviation; SPSS, Statistical Package for the Social Sciences; UPLC, Ultra-Performance-Liquid Chromatography; VT, Voltage transformers.

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مستخلص بذور الكراوية والإرضاع: دراسة في الجسم الحي

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ملخص

تهدف هذه الدراسة إلى التحقيق في تأثير مستخلص بذور الكراوية المائي على إنتاج الحليب لدى إناث الجرذان، وتحديد مكوناته الحيوية الفعالة. تم استخدام ثلاث مجموعات من الجرذان العذارى وثلاث مجموعات من الجرذان المرضعات، بحيث تحتوي كل مجموعة على ست جرذان. تم إبقاء كل أم مع صغارها الستة بشكل منفصل. وابتداءً من اليوم الثالث وحتى اليوم السابع عشر من الرضاعة، تلقت هذه المجموعات يوميًا عن طريق الفم الماء المقطر، أو دواء الميتوكلوبراميد (5 ملغم/كغم)، أو مستخلص بذور الكراوية المائي (200 ملغم/كغم). تم استخدام الطريقة غير المباشرة، التي تربط بين كمية الحليب التي تنتجها الأمهات المرضعات وزيادة وزن الصغار الذين لا يتلقون طعامًا آخر. كما تم قياس مستويات هرمون البرولاكتين لدى إناث الجرذان العذارى في أيام محددة. وفي اليوم الرابع عشر، أظهرت المجموعات التي تلقت مستخلص بذور الكراوية (200 ملغم/كغم) أو الميتوكلوبراميد (5 ملغم/كغم) زيادة ملحوظة في مستويات البرولاكتين (P 0.01 >و P > 0.001على التوالي). وقد بينت الدراسات أن بذور الكراوية تؤدي إلى ارتفاع ملحوظ في البرولاكتين (P 0.05 >في وقت مبكر يصل إلى اليوم السابع. ولكن لوحظ أن الزيادة الملحوظة في إنتاج الحليب وزيادة وزن الصغار كانت فقط في مجموعة الميتوكلوبراميد (5 ملغم/كغم.(P > 0.05) (تشير النتائج إلى أن مستخلص بذور الكراوية يؤثر على مستويات البرولاكتين لدى الجرذان، لكنه لا يؤثر على إنتاج الحليب. وهناك حاجة إلى مزيد من الدراسات لاستكشاف الفوائد العلاجية المحتملة لبذور الكراوية.

الكلمات الدالة: محفز لإدرار الحليب، الكراوية ميتوكلوبراميد، إنتاج الحليب، البرولاكتين، النباتات الطبية.

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Effect of Excipients on the Phenolic Content in Pumpkin Leaf Extracts after Their Introduction into Semisolid Pharmaceutical Forms and Evaluation of In Vitro Stability

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ABSTRACT

The present study aimed to formulate semi-solid pharmaceutical forms from pumpkin leaves (Cucurbita pepo Casper). Total phenolic content was measured after extracting the leaves from Ghouta, Syria. Plant extracts were prepared from Cucurbita pepo Casper leaves using four solvents: ethanol (70%), ethanol, aqueous, and methanol extract. The phenolic content of each extract was quantified. The most effective extracts were incorporated into semi-solid pharmaceutical forms. The influence of excipients on phenolic content was evaluated by measuring phenolic levels in the semisolid formulations and analyzing changes in viscosity and consistency on the shelf. The aqueous extract of Cucurbita pepo 'Casper' leaves exhibited the highest phenolic content, with a concentration of 2.21 ± 11.77 mg/g dry powder. This extract was selected for formulation into three distinct pharmaceutical bases. Among the formulations, the water-in-oil (w/o) cream demonstrated the highest phenolic content and superior stability compared to the oil-in-water (o/w) cream. Stability tests conducted over a three-month period confirmed that the w/o cream maintained optimal stability. Thus, the w/o cream was determined to be the most effective formulation.

Keywords: Cucurbita pepo Casper leaves, aqueous extract, phenolic content, Gallic acid.

1- INTRODUCTION

In recent years, medicinal plants have gained prominence due to their biologically active compounds, which offer various benefits for skincare and overall health. Plant extracts are abundant in vitamins, antioxidants, essential oils, proteins, and other bioactive compounds, providing a range of biological effects including antioxidant, anti-inflammatory, antiseptic, and antimicrobial properties [1-2].

Phenolic compounds are of particular interest due to their ability to neutralize free radicals [3] and their potential in protecting skin from ultraviolet radiation [4]. The composition of phenolic compounds is significantly influenced by the extraction method and solvent used [5].

Cucurbita pepo 'Casper' was selected in this study due to its high carotenoid content, antioxidant capacity, and substantial levels of carbohydrates and minerals, which are beneficial for skin health [6]. Additionally, the plant contains polysaccharides, para-aminobenzoic acid derivatives, sterols, proteins, and peptides, contributing to its medicinal properties, including anti-tumor, immune-enhancing, antibacterial, cholesterol-lowering, anti-parasitic, and anti-inflammatory effects [7-10].

The primary objective of this research was to extract and quantify the phenolic content of Cucurbita pepo

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'Casper' leaves and to incorporate these extracts into semisolid pharmaceutical formulations. The study also aimed to evaluate the impact of excipients on the stability and efficacy of phenolic compounds within these formulations.

Study focus: Cucurbita pepo 'Casper' leaves.

2- MATERIALS AND METHODS:

2-1- Devices and Tools Used: Rising coolant extraction device (Büchi, Flawil, Switzerland), Soxhlet extractor device (Sigma-Aldrich, St. Louis, Missouri, USA), Frosted volumetric flasks 1000 ml, 500 ml, 50 ml (Pyrex, Corning, New York, USA), Sensitive electronic scale AX200 (Shimadzu, Kyoto, Japan), Spectrophotometer device, Hitachi-U-1800(Hitachi, Tokyo, Japan), Mill. Thermometer (IKA, Staufen, Germany), Magra 50 ml and 100 ml (Wilmad-LabGlass, Buena, New Jersey, USA), Frozen micropipettes with different capacities (Eppendorf, Hamburg, Germany), Test tubes - micropipette tips (Gilson, Middleton, Wisconsin, USA), Spectro device T80+ (PG Instruments, Leicester, United Kingdom), Rotary evaporator (IKA, Staufen, Germany).

2-2- Materials:

Cucurbita pepo Casper leaves (Purity: Not applicable (natural material), Farm-grown, Western Ghouta, Damascus, Syria), Distilled water (100% pure distilled water, Generic Laboratory Supply, Syria), Absolute ethyl alcohol (99.5%, Eurolab, London, United Kingdom), Methanol (99.8%, Sigma-Aldrich, St. Louis, Missouri, USA), Anhydrous sodium carbonate (99-100%, PanReac AppliChem, Barcelona, Spain), Standard Gallic acid (98%, Titan Biotech, Rajasthan, India), Folin-Ciocalteu reagent, (Reagent grade, Fluka, Buchs, Switzerland).

2-3- Methods:

2-3-1. Preparation of Plant Samples: Cucurbita pepo* 'Casper' leaves were collected, dried, and ground into a fine powder. Then it was stored in tightly sealed containers to prevent moisture absorption.

2-3-2. Extraction Methods: Four types of extracts were prepared

- Methanolic Extract: 30 g of dried plant powder was extracted with 250 ml of methanol for 2 hours using a Soxhlet extractor. The extract was then concentrated and dried using a rotary evaporator [11].
- Aqueous Extract: 30 g of plant powder was extracted with 200 ml of distilled water, heated under reflux for 1.5 hours, and then evaporated to dryness.[12]
- Ethanolic Extract: 30 g of plant powder was extracted with 300 ml of ethanol for 2 hours. The extract was concentrated and dried using a rotary evaporator.[11]
- Ethanolic 70% Extract: 30 g of plant powder was extracted with 300 ml of a 70/30 mixture of ethanol and distilled water for 2 hours. The resulting extract was dried using a rotary evaporator.[11]

2-3-3 Determination of Total Phenols (T.P.)

The total phenolic content was determined using the Folin-Ciocalteu method. In this process, phenolic compounds reduce the tungsten-molybdate-phosphoric acid complex in an alkaline medium, resulting in the formation of a blue-colored solution. The absorbance of this solution was measured at a wavelength of 760 nm, as a result of a series of reactions where one or two electrons are transferred from the phenolic compounds, leading to the formation of blue-colored complexes.[13]

Preparation of the Standard Series: Gallic Acid Solution: Dissolved 0.5 grams of gallic acid in 10 ml of ethanol, then diluted with distilled water to a final volume of 100 ml in a volumetric flask. The solution can be used daily but is best stored in a closed container in the refrigerator for up to two weeks to prevent degradation.

Preparation of the Calibration Curve: A standard series was prepared by adding 0, 1, 2, 3, 4, 5, and 10 ml of the gallic acid solution to 100 ml volumetric flasks, and then diluting each to the mark with distilled water.

This results in phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L of gallic acid.

Twenty microliters (20 µL) of each standard solution

were pipetted into separate cuvettes. To each cuvette, 1.58 mL of distilled water and 100 µL of Folin–Ciocalteu reagent were added, and the mixture was thoroughly mixed. The samples were incubated for 8 minutes and 30 seconds, after which 300 µL of 20% sodium carbonate solution was added. The mixtures were stirred again and left to stand for 2 hours at 20 °C. Absorbance was then measured at 760 nm using a spectrophotometer, with distilled water serving as the blank. [14]

Preparation of Sodium Carbonate Solution: To prepare a 20% sodium carbonate solution, 200 grams of anhydrous sodium carbonate was dissolved in 800 ml of distilled water. The solution was boiled, cooled, and left to stand for 24 hours before being filtered and diluted to a final volume of 1 liter.[13]

The analysis was conducted in triplicate, and the average values were calculated along with the standard deviation.

2-3-4. Preparation of Pharmaceutical Forms: Three semisolid formulations were prepared: water-in-oil (w/o) cream, oil-in-water (o/w) cream, and hydrophilic cream. Each formulation was analyzed for phenolic content, viscosity, consistency, and stability.

Table (1): Pharmaceutical Formulations (content and ingredients table to follow).

the number	Materials	Formula1	Formula2	Formula 3
1	stearic acid	-	3%	10.5%
2	Tri ethanolamine	-	-	2.5%
3	PolyethyleneGlycol200	40%	-	-
4	Polyethylene glycol 4000	25%	-	-
5	beeswax	-	5%	
6	OgreCytosteryl	-	22%	7%
7	Paraffin oil	-	5%	15%
8	Glycerin	-	10%	15%
9	The spans80%	-	5%	-
10	Preservative	0,1%	0,1%	0,1%
11	Almond oil	-	15%	15%
12	Aqueous extract	35%	35%	35%

Preparation Methods:

A. Hydrophilic Cream (Formula 1):

Polyethylene glycol 4000 was melted in a water bath. Polyethylene glycol 200, the aqueous extract, and the preservative were then added and mixed continuously until the desired cream consistency was achieved.

B. Water-in-Oil (w/o) Cream (Formula 2):

Stearic acid was melted, followed by the addition of beeswax and cetostearyl alcohol. Paraffin oil, almond oil, and sulfur 80 were incorporated at 70°C. Concurrently, the aqueous phase was prepared by dissolving the preservative and glycerin in the aqueous extract. This aqueous phase was then added to the oil phase with continuous stirring in a water bath until the mixture cooled (room temperature).

C. Oil-in-Water (o/w) Cream (Formula 3):

Stearic acid was heated in a water bath until fully melted, after which triethanolamine was added. The remaining ingredients (beeswax, cetostearyl alcohol, paraffin oil, almond oil) were heated to 70°C. This mixture was then combined with the aqueous extract, which had

been mixed with glycerin and heated to 75°C. Stirring continued gradually until the mixture cooled.

2-3-5- Determination of Phenolic Content in Prepared Pharmaceutical Forms:

The phenolic content of each formulation was determined by weighing 1 g of each cream, dissolving it in 100 ml of phosphate buffer (pH 5.4), and stirring the mixture for one hour using a magnetic stirrer. The mixture was then centrifuged at 4500 rpm for 10 minutes, and the supernatant was filtered through a 0.45-micron filter. Optical absorption was measured at 760 nm, and the phenolic content was quantified using a standard curve for the extract. The analysis was performed in triplicate, and the average value along with the standard deviation was calculated.

2-3-6- Statistical Studies:

Statistical analysis was performed on the experimental data using GraphPad Prism software (GraphPad Prism, San Diego, USA). The analysis involved conducting a one-way ANOVA test to assess whether there were significant differences among the groups being compared. After the one-way ANOVA test, Tukey's post-hoc test was applied to determine which specific groups differed from each other. A p-value of less than 0.05 was considered to indicate a statistically significant difference between the groups.

3- RESULTS

3-1- Phenolic Titration Results in Plant Extracts:

Table 2 shows the gallic acid content in various extracts, with mean values and standard deviations, The letters indicate which values are statistically different from each other

Table 2 presents the results of phenolic titration for the four extracts of Cucurbita pepo 'Casper' leaves.

extract type	Ethanol extract	Ethanolic extract70%	Methanolic extract	Aqueous extract
Gallic acid content (sd)	11.10 ± 4.67^{b}	11.57 ± 2.06^{a}	11.07 ± 3.34^{b}	11.77 ± 8.43^{a}

Notes: Values are expressed as milligrams of gallic acid per gram of dry powder ± standard deviation (± sd).

Similar letters indicate that the statistical differences are not significant The different letters indicate that the statistical differences are significant, and the P value was relied upon to indicate the statistically significant difference.< 0.05

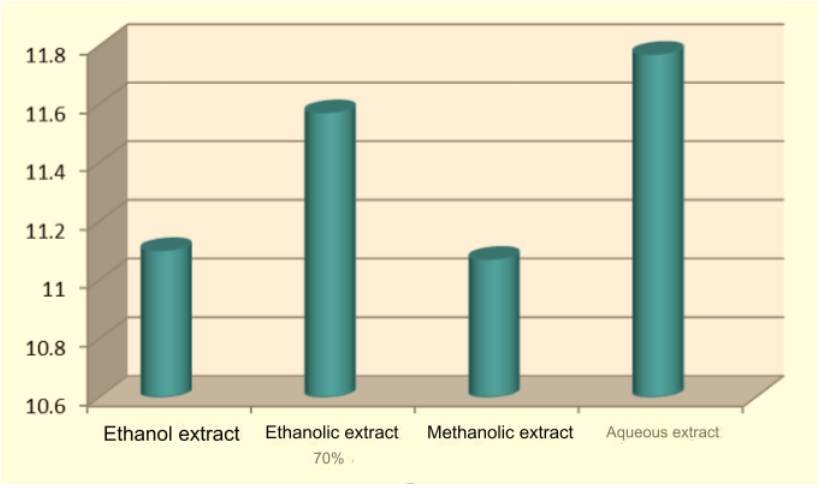

Figure (1) flowchart Phenols in extracts in four casper leaves

Table (3): Average readings of the standard series for Gallic acid

the concentration mg/L	300	250	200	150	100	50	0
Average absorption	0.86	0.72	0.59	0.49	0.31	0.16	0

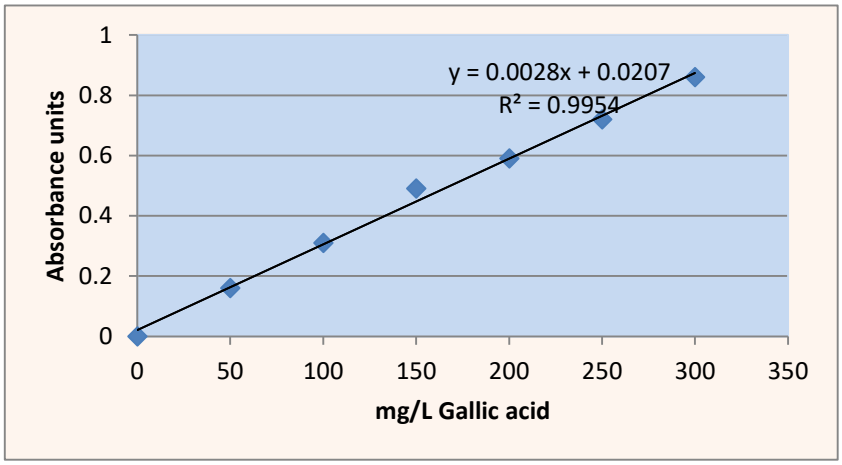

Figure 2: Graphical Curve for Gallic Acid Titration

The graphical curve illustrates the relationship between Gallic acid concentration and its absorbance. The results indicate that the aqueous extract of Casper leaves exhibited the highest gram equivalents of Gallic acid (mg/L) compared to the other extracts.

3-2- Results of the Effect of Excipients on the Phenol Titration in Prepared Pharmaceutical Forms: Table 4 presents the assay results for the phenolic content in the three prepared pharmaceutical forms.

Table 4: Gram Equivalent of Gallic Acid in the three Pharmaceutical Forms

Prepared pharmaceutical form	**Gram equivalent of gallic acid%**
Formula 1	**$\pm$3.64^{b}76**
Formula 2	**$\pm$2.42^{a}92**
Formula 3	**$\pm$1.64^{a}86**

Similar letters indicate that the statistical differences are not significant The different letters indicate that the statistical differences are significant, and the P value was relied upon to indicate the statistically significant difference.< 0.05

3-3- Test Results on the Prepared Pharmaceutical Forms:

3-3-1-Viscosity Examination of the Prepared Pharmaceutical Forms:

This test was performed to assess the ease of application of the prepared pharmaceutical forms at a temperature of 24°C using a viscometer. The results are summarized in Table 5.

Table 5: Viscosity of Prepared Pharmaceutical Forms

Viscosity±SD (Santi Boaz)	The type of pharmaceutical form prepared
2863±23.62 [a]	Formula 1
3266± 17.96 [b]	Formula2
3244±17.962[b]	Formula3

Similar letters indicate that the statistical differences are not significant As for the different letters, they indicate that the statistical differences are significant, and we relied on considering the P value to indicate the statistically significant difference.< 0.05.

Viscosity Results: The viscosity of the prepared pharmaceutical formulations was deemed appropriate for their ease of application on the skin. Formula 1 exhibited the lowest viscosity, while Formula 2 (w/o cream) displayed the highest viscosity.

3-3-2- pH Measurement Results:

Table 6 provides the pH values of the prepared pharmaceutical formulations.

Table 6: pH Values of Prepared Pharmaceutical Forms

The value of splendor pH	Type of pharmaceutical form prepared
5.08 ± 0.22 [b]	Formula 1
5.7±1.02[b]	Formula2
7.4±2.12[a]	Formula3

Similar letters indicate that the statistical differences are not significant The different letters indicate that the statistical differences are significant, and we relied on considering the P value < 0.05 to indicate a statistically significant difference.

pH Results: The pH values of the prepared pharmaceutical formulations were within acceptable ranges, closely matching the skin's natural pH (5.0-5.5), with the exception of the third formulation.

3-3-3- Shelf Stability Examination Results:

The three cream formulations were assessed for stability over one, two, and three months at 25°C. Throughout the testing period, the formulations demonstrated consistent homogeneity, hardness, consistency, and pH stability, indicating that all formulations maintained their stability.

4- DISCUSSION:

The study established that Cucurbita pepo 'Casper' leaves exhibit the highest phenolic content in the aqueous extract, followed in descending order by the 70% ethanolic extract, the ethanolic extract, and the methanolic extract. The observed differences in extraction efficiency are likely due to the interaction between the chemical properties of phenolic compounds and the extraction solvents. The solubility of phenolic compounds is influenced by the polarity of the solvent and the degree of ionization [15]. The aqueous extract exhibited the highest phenolic content, significantly surpassing that of 70% ethanol, likely due to its ability to extract a broader range of active compounds as a result of its polarity.

The aqueous extract was formulated into three distinct pharmaceutical bases, each incorporating different excipients. Among these, the water-in-oil (w/o) cream formulation preserved the highest phenolic content, followed by the oil-in-water (o/w) cream. The hydrophilic cream exhibited the lowest phenolic content. This underscores the crucial role of excipients in maintaining phenolic concentrations within pharmaceutical formulations.

In the formulation containing polyethylene glycol 200 and 4000 (Formula 1), a moderate level of phenolic content was observed. The reduction in phenolic concentration in this formulation may be attributed to the chemical reactivity of polyethylene glycol [16], which can enhance oxidative processes due to the presence of peroxides and secondary oxidation products. This oxidative activity may compromise the efficacy of the phenolic compounds and influence the formulation's physical properties, as polyethylene glycol increases mixture fluidity, potentially leading to phenolic degradation.

The w/o cream (Formula 2) (cream w/o) emerged as the most effective formulation, demonstrating superior phenolic content retention. The protective effect of the oil phase minimized phenol oxidation, and comprehensive stability, viscosity, and pH assessments confirmed its suitability for dermal applications. The excipients used in this formulation were compatible with phenolic compounds, facilitating optimal preservation.

In contrast, the o/w cream (Formula 3), while similar to the w/o cream but with the aqueous phase as the external phase, showed some degree of phenol oxidation due to environmental exposure, though it remained more effective than the hydrophilic cream.

These findings highlight the importance of selecting suitable excipients to optimize phenolic content and formulation stability. The w/o cream formulation was identified as the most effective in preserving phenolic compounds, ensuring the desired therapeutic properties such as anti-inflammatory, anti-allergic, antioxidant, tissue-strengthening, and disinfectant effects [17].

5- CONCLUSION:

Among the four extracts of *Cucurbita pepo* 'Casper' leaves, the aqueous extract exhibited the highest phenolic content, followed by 70% ethanolic, ethanolic, and methanolic extracts. The w/o cream formulation was the most effective in preserving phenolic content, showing suitable viscosity, pH, and stability. The o/w cream ranked second, whereas the hydrophilic cream, although functional, showed reduced phenolic content due to excipient incompatibility.

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تأثير المواد المضافة على محتوى الفينول في مستخلصات أوراق القرع بعد إدخالها في الأشكال الصيدلانية شبه الصلبة وتقييم ثباتها المخبري

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ملخص

هدفت هذه الدراسة إلى تحضير أشكال صيدلانية شبه صلبة من أوراق قرع الكوسا (Cucurbita pepo Casper). تم قياس المحتوى الفينولي الكلي بعد استخلاص الأوراق من غوطة دمشق/سوريا. أُعدت المستخلصات النباتية باستخدام أربعة مذيبات: الإيثانول (70٪)، والإيثانول النقي، والمائي، والميثانولي. ثم قُدر المحتوى الفينولي لكل مستخلص. أُدخلت المستخلصات الأكثر فعالية في الأشكال الصيدلانية شبه الصلبة. تم تقييم تأثير المواد المضافة على المحتوى الفينولي من خلال قياس مستويات الفينول في المستحضرات شبه الصلبة وتحليل التغيرات في اللزوجة والتماسك على مر الزمن. أظهر المستخلص المائي لأوراق القرع أعلى محتوى فينولي (2.21 ± 11.77 ملغم/غم مسحوق جاف)، واختير لتحضير ثلاثة قواعد صيدلانية مختلفة. من بين المستحضرات، أظهر كريم زيت/ماء (w/o) أعلى محتوى فينولي وثباتاً متفوقاً مقارنةً بكريم ماء/زيت (o/w). أكدت اختبارات الثبات لمدة ثلاثة أشهر أن كريم w/o حافظ على ثبات مثالي، مما يجعله الصيغة الأكثر فعالية.

الكلمات الدالة: أوراق قرع الكوسا، مستخلص مائي، محتوى فينولي، حمض الغاليك.

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Extraction, Phytochemical Analysis, and Standardization of Oleuropein-Rich Olive Leaf Extracts: A Study Across Diverse Jordanian Regions

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ABSTRACT

This study aims to investigate and standardize olive leaf extract (OLE) from Jordanian olive trees, examining the impact of geographical differences on the extract's characteristics. Olive leaves were collected from Amman, Karak, Ajloun, and Mafraq and processed using a cost-effective hydro-alcoholic extraction method. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC-UV) were employed to identify and quantify oleuropein, the primary phenolic compound. Additionally, the effects of stress conditions (temperature, UV, humidity) on OLE from Karak were evaluated. The highest oleuropein concentration and total phenolic content were found in extracts from Karak. Physical properties, moisture, ash content, heavy metals, minerals, residual solvents, and microbiological purity were assessed for all extracts. The findings highlight that geographical factors such as altitude and rainfall significantly influence the phenolic content of OLE, with Karak yielding the highest quality extract. The study suggests Jordan's potential as a source of high-quality OLE and recommends further research to improve the stability and formulation of these extracts for therapeutic use.

Keywords: Olive leaves extract; Phytochemical analysis; Jordan; Standardization and quality control.

INTRODUCTION

The olive tree (Olea europaea L.) is believed to be the first domesticated fruit tree in the Mediterranean, and it continues to hold significant economic and cultural importance in this region (1). Olive oil is the primary product, with global production reaching approximately 11 million tons annually (1). The cultivation of olive trees also generates a substantial amount of olive leaves as by-products. These leaves are not merely agricultural waste; they are rich in polyphenolic compounds, particularly biophenols, which possess remarkable biological activities (2–6). The broad spectrum of bioactive compounds found in olive leaf extract (OLE) has garnered interest for their potential applications in cosmetics, pharmaceuticals, and as preservatives to extend the shelf life of food products (4,7,8).

Among the polyphenols in OLE, oleuropein is the most abundant and potent antioxidant, typically comprising between 17% and 23% of the total phenolic content (9). Oleuropein's antioxidant properties are primarily attributed to its ability to donate hydrogen atoms to free radicals, effectively neutralizing them and preventing oxidative chain reactions (10). In addition to its antioxidant capacity, oleuropein exhibits a wide range of pharmacological activities, including anti-inflammatory, anti-cancer, anti-viral, anti-microbial, anti-atherogenic, and anti-aging effects (11). These diverse biological activities have sparked increasing scientific interest in the potential pharmaceutical applications of OLE (12,13). The content of oleuropein in olive leaves varies across geographical regions due to differences in climate, soil composition, altitude, and specific cultivation techniques,

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such as irrigation and fertilization, which influence the plant's metabolic responses and secondary metabolite production. Therefore, understanding this variability is crucial for optimizing the therapeutic use of OLE. Reliable analytical methods are essential to accurately quantify oleuropein and other bioactive compounds.

The analysis of olive leaves requires precise methods for the separation, purification, standardization, and identification (both qualitative and quantitative) of their various constituents (14–16). Although the concept of standardization in phytomedicines is relatively recent, it has quickly become essential to ensure the delivery of high-quality botanical products. Standardization ensures that an extract contains a minimum amount of one or more key compounds, often within a specified range. This process is particularly crucial in phytomedicines, where standardization is applicable solely to extracts (17).

Various analytical tools are employed to ensure the quality and consistency of OLEs. High-performance liquid chromatography (HPLC) is widely used for the precise quantification of oleuropein and other phenolic compounds (17). In addition, Thin-Layer Chromatography (TLC) offers a cost-effective method for the preliminary screening and identification of these compounds, allowing for rapid comparison of samples and confirming the presence of specific bioactive compounds before undertaking more detailed quantitative analyses. Together with other techniques, such as mass spectrometry and spectrophotometry, these methods are integral to the quality control process, ensuring the consistency and efficacy of phytochemical extracts (18,19). Numerous studies have investigated the factors influencing the phenolic profile of olive leaves, including leaf age, ripeness, geographical origin, cultivar, phenological stage during sampling, branch proportion on the tree, moisture content, and industrial extraction processes (2). However, there has been no study specifically evaluating the phenolic profile of Jordanian olive leaves.

In this context, our study aims to investigate the extraction, standardization, and quality control of OLEs from Jordanian olive trees, marking the first such comprehensive analysis. To assess the influence of geographical origin and identify the optimal source of olive leaf extract within Jordan, we selected four distinct regions: Amman, Karak, Ajloun, and Mafraq. This study offers a thorough characterization and standardization of olive leaf extracts, providing a foundational basis for the commercialization of Jordanian olive leaf extract. Moreover, our findings could serve as the groundwork for establishing a preliminary certificate of analysis, ensuring product quality and reliability.

Experimental

Handling of Plant Material

In order to observe the effect of the geographical origin, the following four different regions in Jordan were chosen: Amman, Karak, Ajloun, and Mafraq. These regions have different climates, altitudes and total annual precipitation. Fresh olive leaf samples were collected in the middle of April 2018 from different parts of three trees in the same region. All olive trees were of the same age (about ten years old). A taxonomist confirmed the botanical identity of the plant sample. It was authenticated to be *Olea europaea* L. Sativa (Loudon) Arcang, which belongs to the Oleaceae family. All samples were confirmed to be from the same cultivar and variety. A plant specimen from each geographical area was deposited at the University of Petra Herbarium and given the numbers (OL 1-4/2018). Additionally, a voucher specimen number UOP04/2018/Olea europaea of the leaves was kept for future reference. The collected olive leaf samples were dried using a traditional drying method by shading in a dark place at room temperature for about three weeks, and then the dried olive leaf samples were powdered by using a grinding machine (MOULINEX grinder A843, Mexico). The powdered samples were stored at ambient temperature in a dark place until further extraction.

Extraction

Olive leaf powder (100 g) was added to 500 ml of 80%

ethanol (v/v) in a round-bottom flask and refluxed for approximately 4-5 hours at 70ºC (9,13). After refluxing, the extracts were allowed to cool to room temperature for about 1 hour. The cooled extracts were then filtered, and the liquid filtrates were concentrated using a rotary evaporator (HAHNSHIN S&T CO., LTD., Korea) at 70ºC with a rotation speed of 120 rpm under vacuum. The solvent-free OLE was subsequently dried using a freeze dryer system at -52ºC and 0.2 mbar. The resulting dried OLE powder was stored in an opaque glass container in a refrigerator at 2-8ºC until further analysis. The percentage yield (w/w) of OLE from different regions in Jordan was calculated according to Equation 1.

$$\% \text{ Yield} = \frac{\text{weight of dried extract}}{\text{total weight of olive leaves}} \times 100\% \quad \ldots\ldots\ldots\ldots(1)$$

Phytochemical Analysis and Quality Control

Particle Size

Sieve analysis was used to determine the particle size of OLE powder. A sieve shaker (Gilson, USA) with various mesh sizes was used for 10 minutes. The mesh diameter sizes used were 4, 2, 1, 0.5, 0.25, 0.125 and 0.05 mm. D10, D50, D60, and D90 values were determined. The coefficient of uniformity (C_u) was calculated using Equation 2.

$$C_u = \frac{D60}{D10} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

Density Measurement and Flowability Test

The flowability of the OLEs was characterized using the graduated cylinder method to determine bulk and tap density ρ_{bulk} *and* $\rho_{tap,}$ respectively. Then, a powder flowability test was carried out using Carr's Compressibility index (CI) or Hausner ratio (HR) according to Equations 3 and 4.

$$CI = \frac{100 \times (V0 - Vf)}{V0} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

$$HR = \frac{V0}{Vf} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(4)$$

Loss on Drying (LoD)

Loss on drying (LoD) was measured to determine the moisture content of the samples. About 2 g of each sample was weighed and placed in an infrared moisture determination balance (Kett FD-720, Japan) at 180ºC for about 10 minutes.

Ash Content

The residue remaining after ignition of olive leaf extract is the total ash content or ash value. A Muffle furnace (Lenton FURNACES, UK) and crucible were used for this analysis. Crucibles containing the samples of OLEs were placed in the furnace at 600ºC for 24 hr. After the ash was completed, crucibles were cooled in a desiccator, and the total ash was calculated.

Qualitative Analysis of OLE using TLC

OLE fingerprinting was performed using silica gel plates (Merck; Kieselgel 60 F254, 0.20 mm layers). OLE solutions were prepared by dissolving 0.1 g in 1 ml ethanol. Oleuropein was used as the standard reference. TLC was developed using benzene:ethyl acetate:methanol (60:30:10) as a mobile phase. Then, it was visualized under a UV lamp (366 nm and 254 nm). The retention factor (R_f) for oleuropein was finally calculated.

Quantitative Analysis of OLE using HPLC

The high-performance liquid chromatography (HPLC) method was adapted based on a previously published method by R. Jap'on-Luj'an, M.D. Luque de Castro (20). Partial validation was performed in terms of the limit of detection (LOD), the limit of quantification (LOQ), precision (intraday repeatability) and linearity.

A reversed-phase HPLC analysis was used to standardize the extract using oleuropein as a quality marker. Finnigan surveyor system (Thermo Electron Corporation, San Jose, CA, USA) with an autosampler injector. The injection volume (Autosampler Plus) was 20 µl. A hypersil™ BDS C-18 Column (250 mm x 4.6 mm, 5µm) (Thermo Electron Corporation, San Jose, CA, USA) was used at 40 ºC. Detection was carried out at 254 nm (UV-VIS Plus Detector). The mobile phase was a mixture

of acetonitrile:water (2:8 v/v) with 1% acetic acid and KH_2PO_4 (pH 3.0). The flow rate was 1.0 mL/min (LC pump plus).

A calibration curve was obtained using 10, 50, 100, 200, 400, 800 ppm. Each standard solution was injected six times. Regression equations were calculated in the form of y = ax + b, where y and x were the areas under the peak and standard concentrations, respectively. A sample solution (0.5 % w/v) was prepared in a 100 ml volumetric flask by dissolving 0.5 g of each extract in 80 ml of methanol and placed in an ultrasonic bath at 30°C for 40 min. Subsequently, the volume was completed to 100 ml of methanol. Each sample was prepared in duplicates and injected six times.

Quantitative Analysis of OLE using the Folin-Ciocalteau Method

The folin-Ciocalteu method was used for the phenolic quantification assay following the procedure of Beara et al. (21). It was performed in customized 96-well microplates. Elisa reader (GLOMAX multi-detection system) (Promega Corporation, USA) was used. A calibration curve was calculated using pure gallic acid concentrations ranging from 0.01 to 2 ppm with a regression coefficient of 0.9802. The phenolic content was determined by comparing it with the standard calibration curve of gallic acid. The total phenol value was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (DW) (mg GAE/g of DW).

Stress Conditions Effects

The effect of stress conditions—UV light, elevated temperature (40°C), and high humidity (75%)—on olive leaf extract (OLE) from Karak was tested according to ICH guidelines. Karak samples were selected based on their oleuropein and phenolic content. Two grams of OLE were exposed to these stress conditions for 48 hours. The stressed extracts were analyzed by HPLC-UV at 0, 24, and 48 hours. The influence of UV, elevated temperature, and humidity on the OLE was assessed separately using one-way analysis of variance (ANOVA) to compare the means.

Heavy Metals

Heavy metals analysis was implemented by inductively coupled plasma atomic emission spectroscopy (ICPE-9820, Shimadzu, Japan) to measure the amounts of elemental impurities in OLEs. The software used was Smart Analyzer Vision 5.01.0921. The detector was a charge-coupled device (CCD). ICP-AES is used to detect heavy metals class 1 (Lead, Mercury, Cadmium and Arsenic), which have the highest toxicity risk. Heavy metal contents were determined by the following procedure of Pednekar and Raman (22). The calibration curves were obtained based on the results of the intensity of standard solutions with concentrations ranging from 0.01 to 2 ppm. Heavy metals were detected at Pb 220.353 nm, Hg at 194.227 nm, Cd at 226.502 nm and As was detected at 193.759 nm.

Minerals Content

Inductively coupled plasma atomic emission spectroscopy (ICPE-9820, Shimadzu, Japan) was used to measure OLE's minerals (Calcium, Magnesium, Zinc and Iron) content. Mineral contents were determined by following the procedure of Pednekar and Raman (22). The calibration curves were obtained based on the results of the intensity of standard solutions with concentrations ranging from 0.01 to 2 ppm. Minerals were detected at different wavelengths; Ca was detected at 183.801 nm, Mg at 285.213 nm, Zn at 213.856 nm and Fe at 238.204 nm.

Residual Solvent

The residual solvent was estimated by Gas Chromatography (GC-2010, Shimadzu, Japan) on a CP-Wax 58 CB separation column (30 m x 0.53 mm, Chrompack, Netherlands) to measure the amounts of ethanol residue. An FID detector was used. The flow rates of H_2 and air were set at 30 and 300 mL/min, respectively. The temperature of the FID detector and the injection port was set at 285˚C and 225˚C, respectively. Nitrogen (N2) in the flow rate of 2 mL/min was used as the carrier gas. The software used was (Chrom-Card version 1.06 for Trace GC, TermoQuest, Milan, Italy). The calibration

curve was obtained based on the results of the area under the peak of standard solutions. The resulting solutions contained 15.5, 74.8, 149.9, 299.8, 747.9, 1498.9, and 3077.9 ppm.

Determination of Microbiological Purity

The spread plate technique was used to enumerate the microbial contaminants in OLE samples. About 0.6 mg from each sample was weighed aseptically and dissolved into 1 mL of distilled water. About 0.1 mL of each diluted sample was spread aseptically onto MacConkey agar (Mac), Sabouraud Dextrose Agar (SDA) and Salmonella Shigella (SS) agar media plates for the enumeration of enteric Gram-negative bacteria; e.g., *Escherichia coli* and *Pseudomonas aeruginosa*, fungi and yeasts and *Salmonella*, respectively. Inoculated plates were incubated for 48 h at 25ºC. The microbial count was detected.

Statistical Analysis

The results were expressed as means ± standard deviations. The effect of stress conditions on OLE obtained from Karak was analyzed, and all the determinations were carried out in a triplicate. All statistical analyses were carried out using Microsoft Excel (2017). Comparisons of means with respect to the influence of light, elevated temperature (40ºC), and humidity (75%) were carried out and treated separately using one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Yield of Olive Leaves Extract

The percentage yields of olive leaf extracts (OLE) in our study ranged from 21.00% to 27.95%, with the highest yield obtained from Karak and the lowest from Mafraq (Table 1). For comparison, Jamal et al. (2018) reported yield variations from 0.70% to 16.39% for ursolic acid in *Lantana camara L.* leaves, which were attributed to differences in extraction methods (23). In contrast, our study found that the percentage yields across different geographic regions of Jordan were relatively consistent, ranging from 21% to 28%. This suggests that, despite geographical differences, the extraction efficiency was uniformly effective across the regions studied.

Table 1: Characteristics of Olive Leaves Extract from Different Geographic Regions in Jordan.

Region	Amman	Karak	Ajloun	Mafraq
Yield (%)	26.60	27.95	26.40	21.00
Particle size				
D_{90} mm	1.10	0.98	1.10	1.00
D_{50} mm	0.72	0.70	0.70	0.71
D_{10} mm	0.51	0.52	0.50	0.52
D_{60} mm	0.8	0.80	0.80	0.80
C_u	1.57	1.54	1.60	1.54
Density characteristics				
ρ_{tap} (mean±SD)	0.69±0.02	0.76±0.01	0.76±0.02	0.66±0.01
ρ_{bulk} (mean±SD)	0.55±0.02	0.53±0.01	0.62±0.01	0.50±0.01
Compressibility Index and Hausner's Ratio				
HR (mean±SD)	1.266±0.016	1.429±0.020	1.225±0.023	1.325±0.023
CI % (mean±SD)	21.00±0.90	30.00±1.00	18.33±1.53	24.50±1.32
Flow Property	Passable	Poor	Fair	Passable

Region	Amman	Karak	Ajloun	Mafraq
Other characteristics				
Loss on Drying (mean±SD)	3.67±0.096	3.76 ±0.122	4.48±0.170	4.71±0.043
Ash Content (%)	1.76 %	1.63 %	2.99 %	2.43 %
Total Phenolics (mg of GAE/g of DW) (mean±SD)	14.17±1.17	30.29±0.25	21.32±0.39	7.56±1.02
Ethanol Content (ppm) (mean±SD)	190±1.65	768±11.67	659±13.67	363±3.44
Mineral content				
Ca (mg/g)	0.424	0.464	0.335	0.305
Mg (mg/g)	0.713	0.900	0.530	0.401
Zn (mg/g)	0.010	0.003	0.005	0.006
Fe (mg/g)	0.009	0.007	0.005	0.010

C_u: coefficient of uniformity, ρ_{tap}: tapped density, ρ_{bulk}: bulk density, HR: Hausner ratio, CI: Carr's index, Ca: calcium, Mg: magnesium, Zn: zinc, Fe: iron

Phytochemical Analysis and Quality Control

Particle Size

Particle size results, represented by D10, D50, D90 and D60, and coefficient of uniformity (C_u), are shown in Table 1. Particle size analysis was conducted using sieving, a common method valued for its simplicity, low cost, and minimal expertise requirements (24). The particle size of plant extracts can significantly influence their bioactivity. For example, Hussain et al. found that reducing the particle size of *Vinca rosea L.* extracts from 2.007 ± 0.965 µm to 0.753 ± 0.227 µm improved solubility and enhanced antidiabetic activity (25). In contrast, our study observed that the particle size of OLE was larger, with a D50 value of 0.7 mm. This larger size may be attributed to the use of a commercial-scale grinding machine, suggesting a need for more efficient micronization to meet desired specifications. Despite this, the particle size analysis confirmed that all samples had a uniform grading (26), as indicated by C_u values ranging from 1.54 to 1.6.

Bulk and Tap Density

The bulk density of olive leaf extract (OLE) ranged from 0.50 to 0.62, meeting Ph. Eur. and USP specifications (Table 1). Carr's compressibility index (CI) and Hausner's ratio (HR) were calculated to assess powder flowability. OLE from Amman and Mafraq demonstrated passable flowability, while OLE from Ajloun and Karak showed fair and poor flowability, respectively. Both bulk and tapped densities, used to determine flowability via HR and CI, are influenced by particle size and moisture content. However, the combined effect of these factors on flowability is complex and not fully established, complicating comparisons based solely on physical properties (27). Given the multidimensional nature of flow behavior, no single test can fully capture it. Therefore, adding flowability enhancers is recommended for future formulations, even though the bulk density of OLEs meets USP and Ph. Eur. standards.

Loss on Drying (LoD)

Loss on Drying (LoD) was measured to determine the moisture content of olive leaf extract (OLE) (Table 1). All results were below the 5% acceptance limit specified by the European Pharmacopeia (Ph. Eur., 2007). LoD quantifies moisture content, including both water and volatile matter, which must be minimized in crude drugs (28,29). A study by Kaskoos (2018) reported a higher LoD

of 12.41% for OLE obtained in Iraq using reflux extraction with water (30). This difference may be attributed to variations in extraction solvents. Nonetheless, the LoD results in our study were within the acceptable range, confirming the compliance of our samples with established specifications.

Ash Content

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Qualitative Identification of OLE

TLC fingerprinting was employed for the qualitative identification of oleuropein in OLE, using oleuropein as a standard reference. TLC was investigated at 254 nm (Figure 1A) and 366 nm (Figure 1B). The choice of TLC for this study was driven by its effectiveness in separating and identifying compounds in plant extracts. The Rf value obtained for oleuropein was relatively low (0.16), which is consistent with its known properties under the selected solvent system. While this low Rf value provided a clear identification of oleuropein, it also suggested the need for careful consideration of the mobile phase composition. A stronger mobile phase could potentially enhance the separation of other phytochemicals present in OLE, but it could also result in overlapping spots or reduced specificity for oleuropein. Therefore, the current method was optimized to balance resolution and specificity, particularly for oleuropein. However, for more comprehensive analysis in future studies, slight adjustments to the mobile phase or the use of complementary techniques could be considered to improve separation without compromising specificity.

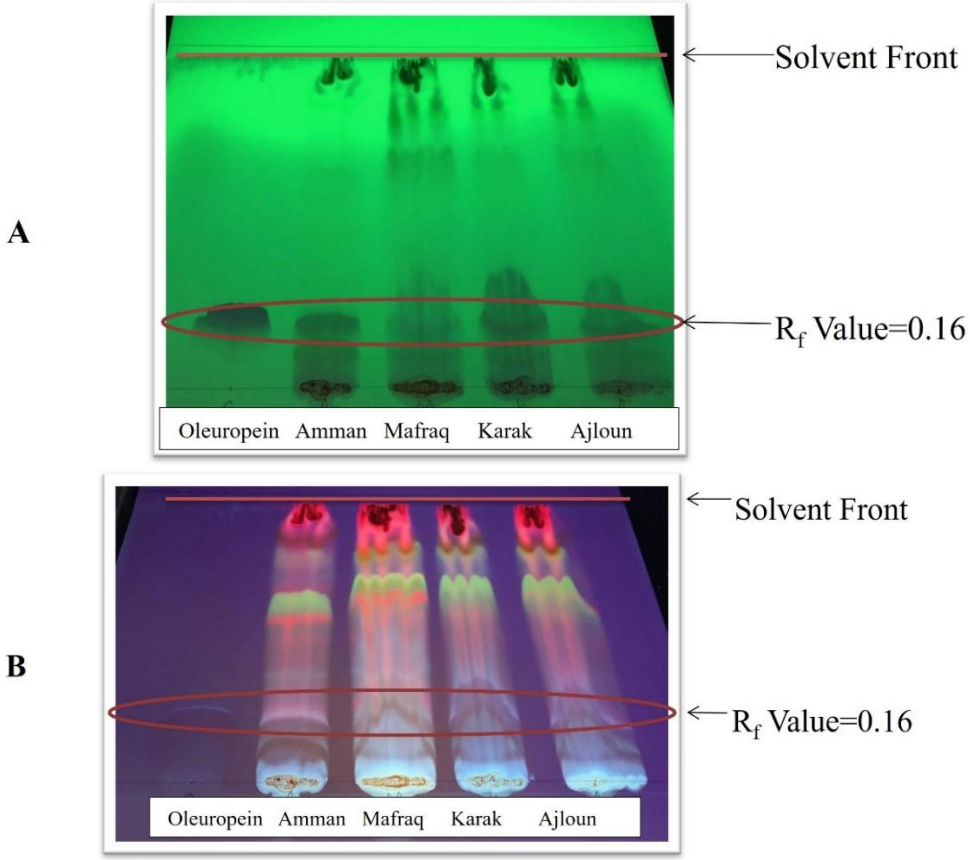

Figure 1: TLC Fingerprinting of Olive leaf extracts at (A) 254 nm and (B) 366 nm.

Quantitative Analysis of OLE using HPLC

High-Performance Liquid Chromatography (HPLC) was employed to quantify oleuropein in OLEs. A calibration curve of oleuropein was plotted (Figure 2) with an R^2 of 0.999. Oleuropein peaks appeared at a retention time of 4.8 min. HPLC assay results revealed notable regional variations (Figure 3). Karak, with its high altitude and substantial annual rainfall, had the highest oleuropein content (19.27%), while Mafraq had the lowest (3.98%). Our results place Karak's oleuropein content above that reported in Turkey (13.4%) (33) but below the levels found in Greece (26.1%) (34). These variations are likely due to differences in climate, soil composition, and altitude.

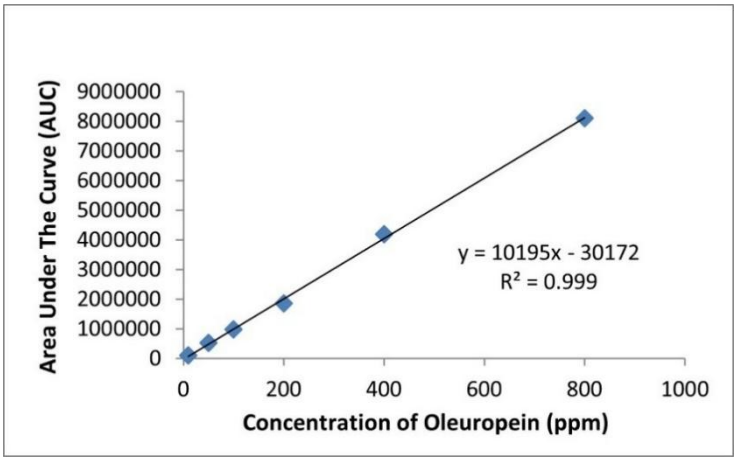

Figure 2: HPLC Calibration Curve of Oleuropein.

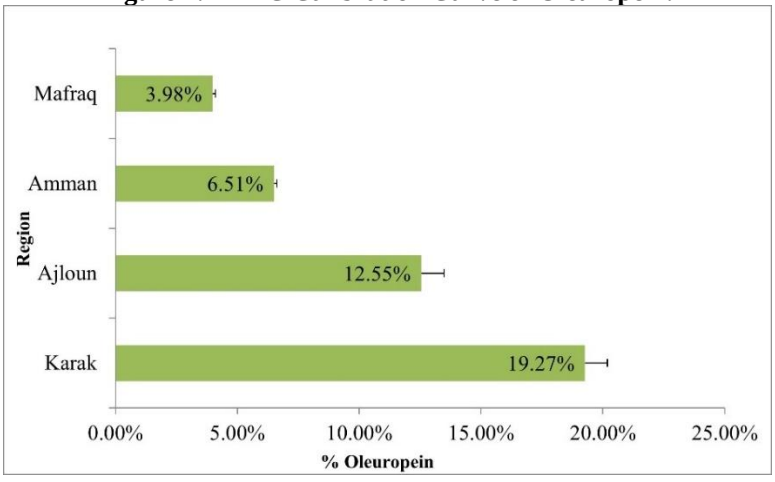

Figure 3: HPLC assay of OLE from Different Geographic Regions in Jordan Represented by Oleuropein.

Quantitative Analysis of OLE using the Folin-Ciocalteau Method

In addition to HPLC, our study employed the Folin-Ciocalteu colorimetric assay to measure the total phenolic content in olive leaves (35). A calibration curve of gallic acid was plotted (Figure 4). This rapid and widely used method revealed significant regional variations in phenolic content across Jordan. The total phenolic content ranged

from 7.56 mg GAE/g DW in Mafraq to 30.29 mg GAE/g DW in Karak (Table 1). This highlights the impact of regional environmental factors, such as soil characteristics, atmospheric conditions, and farming techniques, on the phenolic content.

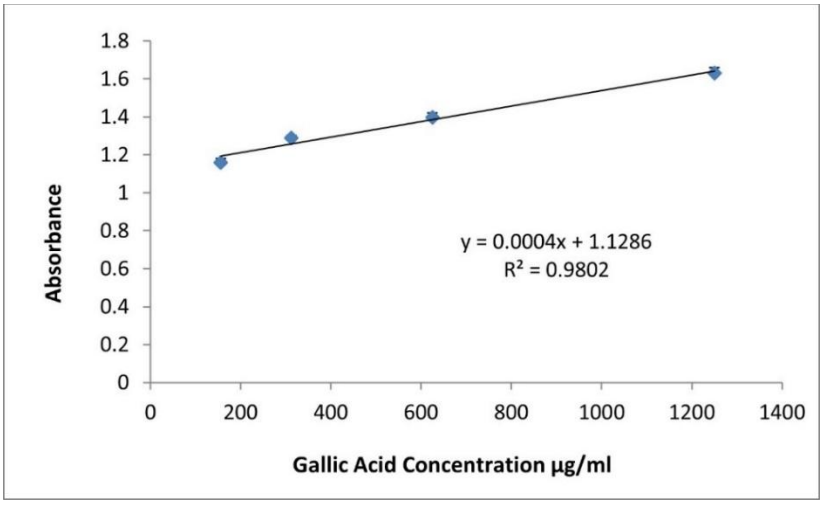

Figure 4: Calibration Curve of Gallic Acid.

Stress Conditions Effects

The effect of stress conditions (UV exposure, elevated temperature of 40ºC, and humidity at 75%) on olive leaf extract (OLE) from Karak (which had the highest oleuropein and phenolic content) was tested (Figure 5). Significant differences were observed under all stress conditions, with a p-value < 0.01, except for the conditions of humidity and elevated temperature after 24 hours, which showed significant differences with a p-value < 0.05. The reduction in oleuropein content exceeded 10% of the initial value.

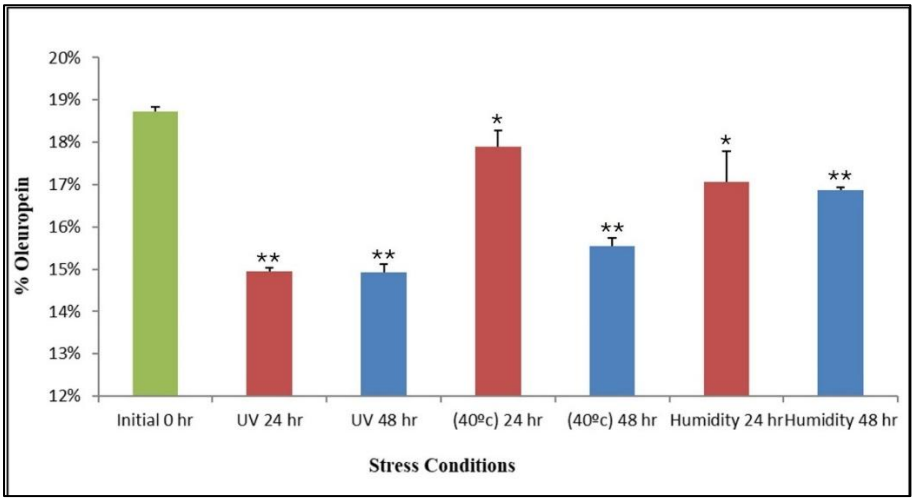

Figure 5: The Effects of Different Stress Conditions on Oleuropein Content in OLE from Karak. *P value <0.05, ** P value <0.01 (level of significance)

Minerals Content

Minerals are essential inorganic substances found in all body tissues and fluids, playing critical roles in maintaining bone health, blood coagulation, acid-base balance, enzyme activity, and osmotic regulation (36). The presence of these minerals in olive leaf extracts (OLE) can support their use for medicinal purposes and as dietary supplements (37). In our study, we measured the levels of calcium, magnesium, zinc, and iron in OLE (reported in Table 1). Our results align with those of Amin et al. who reported similar mineral profiles in their analysis (38). However, research from Brazil evaluating various types of OLE found no significant correlation between phenolic compounds and mineral content (37). This observation is consistent with our findings for OLE from Karak, which exhibited the highest levels of oleuropein and total phenolics, as well as elevated calcium and magnesium concentrations, while zinc and iron levels were relatively low.

Heavy Metals

Heavy metals, even at trace levels, can pose significant risks to human health and the environment (39). Therefore, assessing heavy metal content is crucial in the quality control of medicinal plants to ensure their safety and efficacy (22). In our study, the ICP-AES technique was utilized for its high sensitivity and efficiency in multi-elemental analysis. This method has been recently applied to various plant extracts, as demonstrated by Amin et al. (2016), who used ICP-AES to screen sixty-five elements in Iris persica L. from the Kurdistan region (38). Our analysis of olive leaf extracts (OLEs) from different regions in Jordan revealed that levels of heavy metals, including lead (Pb), cadmium (Cd), and arsenic (As), were within the acceptable limits set by USP and Ph. Eur (Table 2). Mercury was not detected in any OLE samples. Specifically, lead and cadmium were found in OLE from Amman, with concentrations of 0.0109 mg/kg and 0.0006 mg/kg, respectively. Arsenic was present in OLE from Mafraq (0.0114 mg/kg) and Amman (0.0496 mg/kg), but not in Karak or Ajloun. These findings may reflect environmental factors, such as pollution from road traffic and industrial emissions in Amman, which could contribute to higher heavy metal concentrations (40).

Table 2. Heavy Metals Content (ppm) in Olive Leaves Extract from Different Geographic Regions in Jordan.

Region	Pb (ppm)	Pb Specification*	Hg (ppm)	Hg Specification*	Cd (ppm)	Cd Specification*	As (ppm)	As Specification*
Amman	0.0109	≤1.00 ppm	n/d	≤1.00 ppm	0.0006	≤0.50 ppm	0.0496	≤1.00 ppm
Karak	0.0026		n/d		n/d		n/d	
Ajloun	n/d		n/d		n/d		0.0029	
Mafraq	n/d		n/d		n/d		0.0114	

*According to USP and Ph. Eur.

Residual Solvent

Ethanol content in the olive leaf extract (OLE) was evaluated and reported in Table 1. According to the United States Pharmacopeia (USP) 40, 2019, all residual solvent levels were below the acceptance limit. Residual solvents from the extraction process may not be completely removed by standard manufacturing techniques, often resulting in trace amounts remaining in the final product (41). Ethanol, classified as a class 3 residual solvent (42), is commonly used in extraction due to its low toxicity. The USP specifies a maximum allowable residual ethanol level of 5000 ppm in plant extracts. In our study, all samples met this standard, confirming their safety for use.

Determination of Microbiological Purity

Medicinal plant materials often harbor significant amounts of bacteria and molds from the soil, which may

pose pathogenic risks (28). Therefore, assessing microbial contamination is a crucial quality control measure to ensure the safety of olive leaf extract (OLE). The microbial enumeration results, as detailed in Table 3, showed that all OLE samples met the required specifications, confirming they are free from harmful levels of microorganisms.

Table 3. Microbial Count in Olive Leaves Extract from Different Geographic Regions in Jordan.

Microbiological Examination	Specification*	Results
Total Aerobic Microbial Count (cfu/g)	≤5000	Complies
Total Combined Yeast/Molds Count (cfu/g)	≤100	Complies
Pseudomonas Aeruginosa/g	Absent	Complies
Escherichia Coli/g	Absent	Complies
Salmonella/25g	Absent	Complies

*According to Ph. Eur. 5.1.8

DISCUSSION

Olive leaves *(Olea europaea L.)* are a significant by-product of olive cultivation, known for their rich array of polyphenolic phytochemicals with notable biological and pharmacological properties (2,43). Despite Jordan's extensive olive cultivation, with approximately 12 million trees and a strong tradition in both ethnomedicine and rational therapy (44), there has been a lack of studies focusing on the standardization and quality control of olive leaf extract (OLE) in the country. This study represents the first effort to explore the standardization of Jordanian OLE, assess quality control methods through phytochemical analysis, and identify efficient sources within Jordan.

Various extraction techniques, including microwave-assisted extraction, ultrasonication, pressurized liquid extraction, and supercritical fluid extraction, have been used to extract compounds from olive leaves, though these methods can be costly (45). Conventional extraction methods remain preferred due to their cost-effectiveness and efficiency in extracting polyphenolic compounds (46,47). Our study employed a conventional, cost-effective hot extraction method, which allowed for a detailed analysis of the phenolic content and oleuropein yield. The results from this method were then analyzed to assess the quality and potency of the olive leaf extracts. Previous studies have highlighted the impact of extraction parameters such as solvent type, composition, and temperature on phenolic content. For instance, Yateem, Afaneh, and Al-Rimawi (9) identified 80% ethanol as the most effective solvent for extracting oleuropein, while Wissam et al. (48) found that higher temperatures improved extraction efficiency. These findings guided our choice of extraction conditions and helped frame the results of our analyses.

Thin Layer Chromatography (TLC) was utilized for the qualitative identification of oleuropein in OLE, with an Rf value of 0.16. TLC is a widely used technique for phytochemical screening due to its simplicity and effectiveness (30). The low Rf value obtained aligns with the known properties of oleuropein under the selected solvent system. However, the method's resolution and specificity could be improved by adjusting the mobile phase or using complementary techniques in future studies.

HPLC was used in our study to quantify oleuropein in OLE, revealing significant regional variations. The highest oleuropein content, 19.27%, was found in OLE from Karak, a region noted for its high altitude and substantial annual rainfall, which likely enhances oleuropein synthesis. In contrast, OLE from Mafraq had the lowest

oleuropein content at 3.98%. This variation is consistent with Bilgin's (2013) findings that high-altitude regions in Turkey, such as Bursa and Mardin, also show elevated total phenolic content (49). Our results highlight the significant impact of regional environmental factors on oleuropein content in OLEs and underscore the need for regional standardization to ensure consistent quality. Future research should focus on understanding the specific environmental and genetic factors that most significantly affect oleuropein production, to further optimize content across various growing conditions.

The Folin-Ciocalteu colorimetric assay revealed significant regional variations in total phenolic content, with values ranging from 7.56 mg GAE/g DW in Mafraq to 30.29 mg GAE/g DW in Karak. This variation correlates with oleuropein content reported by HPLC, indicating that Karak's olive leaves are particularly rich in both total phenolics and oleuropein. While total phenolic content in Jordanian olive leaves, especially from Karak, is competitive with reported levels from Egypt (34.4 to 39.2 mg GAE/g DW) (50), it is slightly lower.

These findings highlight the significance of geographical origin in determining the quality and potency of OLEs and underscore the need for regional standardization protocols to ensure consistent product quality. Future research should explore how varying environmental conditions and genetic factors, such as genetic variation among olive cultivars, genotype-phenotype relationships, and selective breeding, affect oleuropein production. This investigation could offer valuable insights for optimizing production strategies. Additionally, future studies should assess the impact of different extraction methods and solvents on phenolic profiles to further enhance the understanding of their effects.

5. CONCLUSION

Olive leaves are a valuable but underutilized source of phenolic compounds. This study is the first to standardize olive leaf extract (OLE) from various regions in Jordan using a cost-effective extraction method with 80% aqueous ethanol. Phytochemical and quality control methods were applied to leaves from Amman, Karak, Ajloun, and Mafraq, all of which met USP and Ph. Eur. specifications.

The results revealed that OLE from Karak exhibited the highest levels of oleuropein and total phenolic content. Karak's Mediterranean climate, high altitude, and substantial rainfall are likely contributors to this superior quality. Therefore, controlled cultivation of olive trees in Karak is recommended. This research establishes a foundation for the commercialization of Jordanian OLE and underscores Jordan's potential as a rich source of phenolic and antioxidant compounds from its extensive olive groves. Future research should focus on enhancing extract stability and developing appropriate dosage forms.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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إعداد وتوحيد المستخلصات وتحليل المكونات الكيميائية النباتية لأوراق الزيتون (Olea europaea L) من مناطق جغرافية مختلفة في الأردن

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ملخص

تهدف هذه الدراسة إلى التحقيق في استخلاص وتوحيد مستخلص أوراق الزيتون (OLE)من أشجار الزيتون الأردنية، مع دراسة تأثير الاختلافات الجغرافية على خصائص المستخلص. تم جمع أوراق الزيتون من عمان والكرك وعجلون والمفرق، وتمت معالجتها باستخدام طريقة استخلاص كحولية مائية منخفضة التكلفة. تم استخدام تقنية الكروماتوجرافيا الرقيقة (TLC)والكروماتوجرافيا السائلة عالية الأداء (HPLC-UV)لتحديد وقياس الأوليوروبين، وهو المركب الفينولي الرئيسي. بالإضافة إلى ذلك، تم تقييم تأثير ظروف الإجهاد (الحرارة، الأشعة فوق البنفسجية، الرطوبة) على المستخلص من الكرك. أظهرت النتائج أن أعلى تركيز للأوليوروبين وأعلى محتوى فينولي كلي كان في المستخلصات من الكرك. تم تقييم الخصائص الفيزيائية والمحتوى الرطوبي ومحتوى الرماد والمعادن الثقيلة والمعادن والمذيبات المتبقية والنقاوة الميكروبيولوجية لجميع المستخلصات. أظهرت النتائج أن العوامل الجغرافية مثل الارتفاع وهطول الأمطار تؤثر بشكل كبير على المحتوى الفينولي لمستخلص أوراق الزيتون، مع حصول الكرك على أفضل مستخلص من حيث الجودة. تشير الدراسة إلى إمكانية الأردن كمصدر لمستخلص أوراق الزيتون عالي الجودة، وتوصي بإجراء المزيد من الأبحاث لتحسين استقرار وتشكيل هذه المستخلصات للاستخدام العلاجي.

الكلمات الدالة: مستخلص أوراق الزيتون؛ التحليل النباتي الكيميائي؛ الأردن؛ التوحيد ومراقبة الجودة.

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Impact of Oxidative Stress on Jordanian Children with Autism Spectrum Disorder

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ABSTRACT

Background: Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder whose etiology is still unknown and without clinical biomarkers. Recent studies have highlighted the potential role of oxidative stress and metabolic changes in ASD. However, little is known about these changes in the Jordanian ASD population.

Aims: This study aimed to evaluate oxidative stress biomarkers in Jordanian children with ASD and to investigate the potential correlations with the disorder's clinical features.

Methodology: This cross-sectional study involved 80 Jordanian children divided into two groups: the patients' group (diagnosed with ASD, n=40) and the control group (healthy, n=40). The study examined the distribution of ASD among the participants and assessed the prevalence of comorbid conditions. It also evaluated oxidative stress biomarkers, including Glutathione Peroxidase (GPX), Superoxide Dismutase (SOD), and Malondialdehyde (MDA).

Results: ASD was more common in males (65% in the ASD group) and in people with a family history of the disorder (55%). Common comorbid conditions included ADHD (42.5%), anxiety (25%), and epilepsy (15%). Children with ASD had significantly lower levels of GPX (2.72 ± 0.9 pmol/mL vs. 7.74 ± 2.5 pmol/mL in controls, $p<0.005$) and SOD (1.74 ± 0.75 ng/mL vs. 2.93 ± 0.98 ng/mL in controls, $p<0.005$) and higher levels of MDA (16 ± 1.95 nmol/mL vs. 5.46 ± 1.57 nmol/mL in controls, $p<0.005$).

Conclusion: This study suggests a potential association between ASD and oxidative stress. While further research is required, these findings contribute to our understanding of ASD pathogenesis and may guide future diagnostic and therapeutic approaches. Pearson correlation coefficients imply that increased oxidative stress, as measured by lower GPX and SOD levels and higher MDA levels, may be linked to the severity and presence of clinical features in ASD.

Keywords: Autism Spectrum Disorder, Jordanian children, Glutathione Peroxidase, Superoxide Dismutase, Malondialdehyde, ADHD, anxiety, epilepsy, oxidative stress.

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INTRODUCTION

Autism Spectrum Disorder (ASD) is a multifaceted neurodevelopmental disorder, manifesting during early childhood, with variations in its expression owing to its spectrum nature [1–3]. Although there is limited research on the incidence of ASD in Jordan, it is believed that one out of every 50 children has ASD, giving an approximate total number of 10,000 children with ASD in Jordan, which is considered lower than the prevalence in the well-developed countries [4]. It encompasses many symptoms, impacting social interactions, communication, and more [5,6]. Despite its prevalence, with about 1 in 54 children diagnosed with ASD in the U.S. alone and an estimated two million in the Middle East, a comprehensive understanding of its etiology remains elusive [2,7,8].

Oxidative stress in ASD is linked to metabolic disturbances like lipid peroxidation and mitochondrial dysfunction, causing a vicious cycle that impacts brain development and function [9–11]. The origins of ASD are believed to be rooted in a combination of genetic and environmental factors [12]. Even with over 1,000 genes linked to ASD, none dominate the causality, making its genetic makeup a complex tapestry [13]. Moreover, its frequent coexistence with other disorders, such as intellectual disabilities, compounds the challenge of discerning specific biomarkers [14]. Presently, diagnosis is heavily reliant on behavior-centric evaluations, which can inadvertently lead to delays.

Recently, the realm of oxidative stress and its correlation with ASD has attracted attention [15]. Oxidative stress is a phenomenon where there is an imbalance between free radicals and antioxidants in the body [16–18]. Several studies have shed light on heightened oxidative stress in individuals with ASD, suggesting potential biomarkers for the condition [19,20]. These potential biomarkers might also use for primary analysis and assessment of ASD intervention and notify ASD nutritional and pharmacological treatment interventions [21].

Signs of this stress, such as increased lipid peroxidation and DNA damage, have been discerned in peripheral fluids and have shown correlations with the severity of ASD symptoms [22].

In children, especially, the vulnerability to oxidative stress is more pronounced due to naturally low levels of some antioxidants. Notably, studies have indicated diminished levels of certain antioxidants in children with ASD, which could amplify oxidative stress in their brain cells [23,24].

Furthermore, the relationship between oxidative stress and inflammation, particularly in the brains of those with ASD, provides another avenue of exploration [25,26]. While sampling challenges exist, discoveries from various cell types hint at an underlying association between inflammation, oxidative stress, and ASD [27].

Elevated levels of malondialdehyde (MDA) and altered activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) in people with Autism Spectrum Disorder (ASD) indicate increased oxidative stress and disrupted antioxidant defenses, implying that oxidative stress may play a role in the disorder's pathology [28]. Given the significance of oxidative stress in ASD's framework and the gaps in our understanding, especially in younger populations, this study aims to delve deeper into this connection. Our research seeks to elucidate the role of oxidative stress in the onset of ASD among Jordanian children, emphasizing potential biomarkers like malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPX). By enhancing our grasp on this dimension, we aspire to pave the way for better therapeutic approaches for children grappling with ASD.

MATERIALS AND METHODS

Study design and participants selection

This study was conducted at the International Behavioral Intervention Center in Amman, Jordan. Participants, ranging in age from 4 to 14 years, were divided into two groups: those with a confirmed ASD

diagnosis based on the Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revision (DSM-IV-TR) criteria (ASD group, n=40) and age-matched controls without any history of ASD (Control group, *n*=40).

Selection ensured an unbiased representation from a total pool of 80 children. Comprehensive demographic and medical data, encompassing age, sex, and medical history, were collected for each participant to identify potential variables associated with ASD.

Exclusion criteria were critical in mitigating potential confounding factors. Similarly, children diagnosed with hematological or immunological conditions like anemia, metabolic disorders including diabetes and heart disease, or with existing liver or kidney ailments were excluded. Additionally, any child who had received treatment involving vitamins or antioxidant supplements was not incorporated in the study to eliminate their potential influence on the research outcomes. A specialist in neurology confirmed the diagnosis of ASD.

Blood sampling

Blood samples were procured from case and control group participants for biological assessment. Using standard venipuncture techniques, 5 ml of venous blood was collected from each participant under the guidance of a trained phlebotomist. Upon collection, samples were immediately introduced to EDTA tubes to inhibit clotting. Following this, samples were centrifuged at 3000 RPM for 15 minutes under refrigeration, segregating the plasma from other constituents. The extracted plasma was subsequently transferred to sterile tubes and preserved at -80°C to maintain its integrity until subsequent analyses.

GPX, SOD, and MDA in the plasma samples.

The assay for plasma GPX, SOD, and MDA levels was conducted using the human GPX, human SOD, and human MDA ELISA kits, respectively (Genochem World, Spain), according to the manufacturer's protocol. The concentrations in plasma were inferred from a standard curve.

Ethical approval

Before collecting blood samples, ethical approval was obtained from the Ethical Writing and Scientific Committee of the Faculty of Allied Medical Sciences, Al-Ahliyya Amman University (IRB: AAU/3/12/2022-2023). The study upheld strict confidentiality and maintained the anonymity of all individuals involved.

Statistical analysis

Data were represented as mean ± standard deviation (SD). Mann-Whitney U test was used to analyze the mean difference and determine whether the two groups differed significantly. A *P*-value of 0.05 or lower was interpreted as statistically significant. Statistical analyses were performed using Prism 8 software.

Pearson correlation coefficients were calculated to assess the relationships between oxidative stress biomarkers and clinical features. A p-value < 0.05 was considered statistically significant.

RESULTS

Subject characteristics

In this cross-sectional study, the focus was on evaluating oxidative stress biomarkers in a population of children diagnosed with ASD, using a control group for comparison.

The demographic characteristics of the study participants were meticulously recorded and are summarized in **Table 1**. The age range among participants spanned from 4 to 14 years. The children with ASD had a mean age of 7.5 ± 2.3 years, paralleling closely with the control group, which had a mean age of 7.4 ± 1.6 years.

The ASD group demonstrated the often-documented gender disparity associated with ASD prevalence, comprising 26 males (65%) and 14 females (35%). In contrast, the control group presented a nearly balanced gender distribution, with 18 males (45%) and 22 females (55%).

An influential element observed within the ASD group

was a family history of ASD, which was present in a significant 55% (22 children) of the group compared with the control.

A commonality among children with ASD is the presence of co-occurring conditions or comorbidities. Within the ASD group, the most common comorbidities observed were attention deficit hyperactivity disorder (ADHD) in 17 children (42.5%), anxiety disorders in 10 children (25%), and epilepsy in 6 children (15%).

Table 1. Demographic characteristics of study participants

Variable	ASD *n*= 40	Control *n*= 40
Age (years)	7.5±2.3	7.4±1.6
Gender, n (%)		
Male	26 (65)	18 (45)
Female	14 (35)	22 (55)
Height (cm)	123±6.55	125±8.24
Weight (kg)	27.06±5.3	28.31±7.1
BMI (kg/m2)	17.88±2.5	18.11±3.5
Family history, n (%)	22 (55)	NI
Co-occurring conditions, n (%)		
Epilepsy	6 (15)	NI
ADHD	17 (42.5)	NI
Anxiety	10 (25)	NI

Abbreviations: BMI: Body Mass Index; ADHA: Attention Deficit Hyperactivity Disorder; NI: not investigated.

Evaluating the levels of GPX, SOD, and MDA in children with ASD.

In our study, children with ASD exhibited significantly ($p<0.005$) lower GPX levels (2.72 ± 0.9 pmol/mL) compared to the control group (7.74 ± 2.5 pmol/mL), as illustrated in Figure 1A.

Additionally, SOD levels were evaluated. The ASD group demonstrated a significant ($p<0.005$) decline in SOD activity compared to the control (1.74 ± 0.75, 2.93 ± 0.98 ng/mL, respectively), as presented in Figure 1B.

In contrast to the lowered GPX and SOD, MDA levels, MDA was elevated in the ASD group. Specifically, the ASD group showed a mean MDA level of 16 ± 1.95 nmol/mL, substantially ($p<0.005$) higher than the control's 5.46 ± 1.57 nmol/mL (Figure 1C).

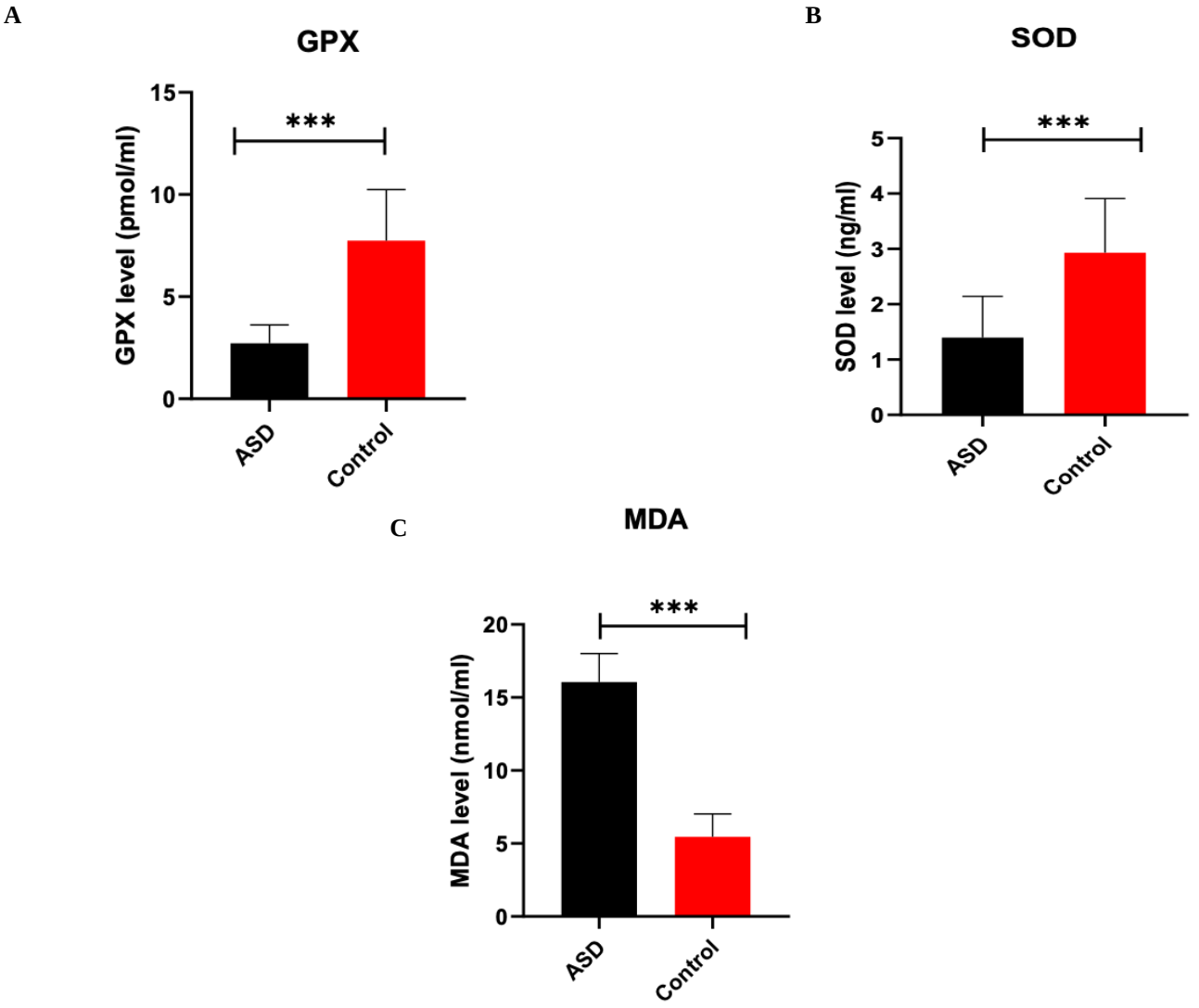

Figure 1. The levels of GPX (A), SOD (B), and MDA (C) in the plasma of children diagnosed with ASD. Data represent mean ± SD. *** denotes a P-value < 0.005 calculated by the Mann-Whitney U test.

Correlations Between Oxidative Stress Biomarkers and Clinical Features in ASD

This section investigates the correlations between oxidative stress biomarkers (GPX, SOD, MDA) and clinical features (age, gender, family history, comorbidities) in Jordanian children with ASD.

The findings of this study indicate a significant correlation between oxidative stress biomarkers and various clinical features in children with ASD. For example, age was found in this study to have substantial negative correlations with GPX and SOD but a positive correlation with MDA. In addition, males were found to have negative correlations with GPX and SOD but positive correlations with MDA. However, females showed the

opposite trend. Family history has shown a negative correlation with GPX and SOD and a positive correlation with MDA. Studying the comorbidities, we noticed that ADHD and anxiety had significant negative correlations with GPX and SOD but positive correlations with MDA. Epilepsy demonstrated similar but weaker trends.

Pearson correlation coefficients were computed to evaluate the associations between oxidative stress biomarkers and clinical characteristics. A *p*-value less than 0.05 was deemed statistically significant, **Table** 2.

Table 2. Correlation coefficients to assess the relationships between oxidative stress biomarkers and clinical features

Clinical Feature	GPX (*r*, *p*-value)	SOD (*r*, *p*-value)	MDA (*r*, *p*-value)
Age	-0.35, 0.02	-0.40, 0.01	0.45, 0.005
Male	-0.30, 0.03	-0.32, 0.02	0.50, 0.001
Female	0.30, 0.03	0.32, 0.02	-0.50, 0.001
Family History	-0.25, 0.05	-0.28, 0.04	0.35, 0.02
Epilepsy	-0.20, 0.10	-0.25, 0.05	0.30, 0.03
ADHD	-0.40, 0.01	-0.45, 0.005	0.55, 0.001
Anxiety	-0.35, 0.02	-0.40, 0.01	0.50, 0.001

DISCUSSION

Autism Spectrum Disorder (ASD) has emerged as a pressing public health concern globally [29]. A complex neurodevelopmental disorder persisting from childhood to adulthood, its etiology remains multifaceted in adulthood [30]. Our study delved into the oxidative stress biomarkers in Jordanian children with ASD.

Oxidative stress is linked to ASD in children, with several biomarkers related to clinical features. For example, elevated levels of MDA, a byproduct of lipid peroxidation, are correlated with the severity of autism symptoms. Also, lower levels of glutathione, a crucial antioxidant, are associated with impaired detoxification pathways and increased vulnerability to oxidative stress. In addition, enzymes like superoxide dismutase and catalase show altered activity in children with ASD, suggesting a disrupted antioxidative defense mechanism. Furthermore, higher oxidative stress markers are associated with more severe behavioral symptoms, including social and communication difficulties. Add to that, the inflammatory markers, such as C-reactive protein, are also linked to ASD [31–34].

In line with global statistics, we observed that we had found not high ASD prevalence among males but higher male prevalence among ASD patients, underscoring established gender disparities in ASD diagnoses [35]. This contrast accentuated the gender-based difference in ASD prevalence, a factor that has been subject to extensive research and interest. While the exact cause remains elusive, the "female protective model" posits that females may have a natural defense against ASD [36]. X-linked ASD-associated genes and hormonal factors, including elevated testosterone levels in males, are also considered contributors to this disparity [37].

Over half of our ASD participants had a familial link to the disorder, emphasizing the significance of genetic factors in ASD [38]. Sandin et al., 2014 and Gaugler et al. 2014 delineated the genetic component of ASD, suggesting a blend of inherited and spontaneous mutations as influential[39]. The Autism Sequencing Consortium further highlighted multiple ASD-associated genes impacting brain functionality [40]. While genetics play a part, environmental factors also likely contribute, creating a complex interplay that culminates in ASD [41]. Technological strides in genetic sequencing promise potential breakthroughs in ASD detection and intervention [42].

Comorbidities were prevalent among our ASD cohort, with notable percentages diagnosed with ADHD, anxiety, and epilepsy. This multifaceted clinical profile mirrors global observations. Our findings on the co-occurrence of ASD with ADHD (42.5%) align with [43]. While the 25% anxiety prevalence in our study is slightly lower than that found by White et al. 2009 [44] findings, it still underscores

the frequent coexistence of these conditions. Lastly, our observation of a 15% epilepsy rate among children with ASD aligns with the broader literature [45].

GPX, a pivotal antioxidant enzyme, plays a significant role in protecting against oxidative damage by neutralizing harmful peroxides. Of paramount importance were the findings regarding oxidative stress biomarkers. ASD children exhibited a significant decrease in the levels of GPX and SOD. Both GPX and SOD are critical antioxidants in the human body. SOD is essential for counteracting superoxide radicals, which, if unchecked, can cause oxidative harm. A decrease in these enzymes suggests an impaired antioxidant defense system in children with ASD, resulting in increased susceptibility to oxidative stress [46]. Such a decrease indicates a compromised defense mechanism against oxidative damage in children with ASD.

Underlining the role that genetic factors may play in the disorder. The presence of this genetic predisposition could potentially influence the severity and presentation of ASD symptoms and oxidative stress levels. Moreover, MDA, a byproduct of lipid peroxidation and a marker for oxidative stress, was significantly increased in the plasma of children with ASD. This increase in MDA levels indicates a higher rate of lipid peroxidation processes, implying the existence of oxidative stress in the body [47].

Several studies in the literature resonate with these findings. A meta-analysis by Frustaci et al. 2012 [48] and a recent one by Chen et al. (2021) confirmed significantly lower levels of antioxidant enzymes, including GPX and SOD, and elevated levels of oxidative stress markers in individuals with ASD compared to controls. Similarly, a study by Nasrallah et al. 2022 [49] found that children with ASD had lower levels of GPX and SOD and higher levels of oxidative stress markers, including MDA.

MDA is a marker of oxidative stress resulting from lipid peroxidation. Increased MDA levels indicate enhanced lipid peroxidation and oxidative damage. This is consistent with the study by Meguid et al. 2011 [50], which reported significantly increased levels of MDA in children with ASD compared to controls.

Several mechanisms have been proposed to explain the link between these oxidative stress biomarkers and ASD. Firstly, increased oxidative stress could lead to neuronal damage, affecting the development and function of the nervous system, which could contribute to the neurodevelopmental abnormalities observed in ASD [51]. Secondly, oxidative stress could induce inflammation, another process thought to be involved in ASD pathophysiology [52]. Finally, there is a growing body of evidence suggesting a genetic component in ASD that might also influence oxidative stress [53]. Certain genetic variations found in individuals with ASD could impact the activity of antioxidant enzymes or the production of ROS, thereby affecting the balance between oxidative stress and antioxidant defenses [54]. Each of these comorbidities may interact with ASD and oxidative stress in complex ways. For instance, stress and anxiety associated with these conditions could potentially elevate oxidative stress levels, underscoring the multifaceted nature of ASD's clinical presentation and pathophysiology.

The ASD group's increased MDA and decreased GPX and SOD levels emphasize elevated oxidative stress. This reinforces the proposed connection between oxidative stress and ASD, warranting further studies on its potential role in ASD's pathophysiology.

These findings, suggesting an association between oxidative stress and ASD, may have important implications for understanding the pathophysiology of ASD.

Oxidative stress, caused by an imbalance between free radicals and antioxidants, is a significant factor in the pathophysiology of ASD. Elevated reactive oxygen species and reactive nitrogen species contribute to oxidative stress in ASD, damaging cellular components like lipids, proteins, and DNA, potentially disrupting brain function [55,56]. Research has shown that oxidative stress markers, such as hydroperoxides and decreased

antioxidant capacity, are significantly higher in individuals with ASD compared to neurotypical controls [55,56]. Antioxidant supplementation has shown promise in reducing oxidative stress and improving behavioral symptoms in individuals with ASD, with nutritional interventions focusing on enhancing antioxidant defenses being explored [57].

They also hint at potential therapeutic interventions to restore antioxidant defenses and reduce oxidative stress. However, it remains unclear whether these alterations are a cause or an effect of ASD, and further research is required to elucidate these relationships and their therapeutic potential in ASD management.

The study's limitations encompass a small sample size, potentially constraining the generalizability of the findings to the wider population of Jordanian children with ASD. In addition, the cross-sectional design precludes the establishment of causal inferences. Furthermore, the control group matching failed to account for other potentially confounding variables. In addition, particular populations, including individuals with comorbidities or those utilizing antioxidant supplements, were omitted from the study. Indeed, the research was performed at a singular location, resulting in site-specific biases. Also, assessing oxidative stress biomarkers in plasma may not entirely represent oxidative stress in the brain. Another limitation is the absence of longitudinal data, which hinders the comprehension of temporal variations in biomarkers. Also, temporary factors can affect biomarker levels. Finally, the research concentrates on Jordanian children, thereby restricting its relevance to other ethnic or genetic groups.

The findings indicate that elevated oxidative stress, evidenced by diminished GPX and SOD levels and increased MDA levels, may correlate with the severity and presence of clinical features in ASD.

CONCLUSION

In summary, we found that children with ASD showed a significant decrease in the antioxidant enzymes GPX and SOD, along with a substantial increase in MDA levels, a marker for oxidative stress, indicating an impaired antioxidant defense system and increased oxidative stress. These findings suggest a link between ASD and an impaired antioxidant defense system, which may heighten the susceptibility to oxidative stress, potentially contributing to the pathophysiology of ASD. The study highlights the potential role of oxidative stress in ASD pathogenesis and its association with clinical features. Further research is needed to explore these relationships and their implications for diagnostic and therapeutic strategies.

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تأثير الإجهاد التأكسدي على الأطفال الأردنيين المصابين باضطراب طيف التوحد

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ملخص

الخلفية :اضطراب طيف التوحد (ASD) هو اضطراب نمائي عصبي لا تزال مسبباته غير معروفة ولا توجد له مؤشرات حيوية سريرية. وقد أبرزت دراسات حديثة الدور المحتمل للإجهاد التأكسدي والتغيرات الأيضية في اضطراب طيف التوحد. إلا أنه لا يُعرف الكثير عن هذه التغيرات لدى المصابين باضطراب طيف التوحد في الأردن.

الأهداف :هدفت هذه الدراسة إلى تقييم الواسمات الحيوية للإجهاد التأكسدي لدى الأطفال الأردنيين المصابين باضطراب طيف التوحد، والتحقيق في الارتباطات المحتملة لهذه الواسمات بالسمات السريرية للاضطراب.

المنهجية :شملت هذه الدراسة المقطعية 80 طفلًا أردنيًا قُسّموا إلى مجموعتين: مجموعة المرضى (شُخِّص أفرادها باضطراب طيف التوحد، وعددهم 40) والمجموعة الضابطة (من الأصحاء، وعددهم 40). تم استقصاء توزيع اضطراب طيف التوحد بين المشاركين وتقييم مدى انتشار الحالات المرضية المصاحبة. كما تم تقييم الواسمات الحيوية للإجهاد التأكسدي، بما في ذلك إنزيم غلوتاثيون بيروكسيداز (GPX) ، وإنزيم سوبر أكسيد ديسميوتاز (SOD) ، ومالونديالدهيد.(MDA)

النتائج :كان اضطراب طيف التوحد أكثر شيوعًا بين الذكور (65% في مجموعة المرضى) وبين الأشخاص الذين لديهم تاريخ عائلي للإصابة بالاضطراب (55%). كما تضمنت الحالات المرضية المصاحبة الشائعة كلاً من اضطراب نقص الانتباه مع فرط النشاط (ADHD)بنسبة 42.5%، والقلق بنسبة 25%، والصرع بنسبة 15%. كانت مستويات إنزيم GPX لدى الأطفال المصابين بالتوحد أقل بشكل ملحوظ (2.72 ± 0.9 بيكومول/مل مقابل 7.74 ± 2.5 بيكومول/مل في المجموعة الضابطة، ($p<0.005$)، وكذلك مستويات إنزيم SOD (0.75 ± 1.74 نانوغرام/مل مقابل 2.93 ± 0.98 نانوغرام/مل في المجموعة الضابطة، ($p<0.005$)، في حين كانت مستويات MDA لديهم أعلى (1.95 ± 5.46 نانومول/مل مقابل 1.57 ± 16 نانومول/مل في المجموعة الضابطة، ($p<0.005$).

الخلاصة :تشير هذه الدراسة إلى وجود ارتباط محتمل بين اضطراب طيف التوحد والإجهاد التأكسدي. وعلى الرغم من ضرورة إجراء المزيد من البحوث، فإن هذه النتائج تسهم في تعزيز فهمنا لإمراضية اضطراب طيف التوحد وقد توجه الأساليب التشخيصية والعلاجية المستقبلية. كما تشير معاملات ارتباط بيرسون إلى أن زيادة الإجهاد التأكسدي، المتمثلة في انخفاض مستويات إنزيمي GPXو SOD وارتفاع مستوى MDA، قد تكون مرتبطة بظهور السمات السريرية وشدتها لدى المصابين باضطراب طيف التوحد.

الكلمات الدالة: اضطراب طيف التوحد، أطفال أردنيون، غلوتاثيون بيروكسيداز، سوبر أكسيد ديسميوتاز، مالونديالدهيد، اضطراب نقص الانتباه مع فرط النشاط (ADHD)، القلق، الصرع، الإجهاد التأكسدي.

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A Comprehensive Novel Stability indicating Method Development and Validation for Simultaneous Assessment of Abiraterone and Niraparib in Bulk and Pharmaceutical Formulation by Ultra Performance Liquid Chromatography

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ABSTRACT

Background: This study aims to develop and validate an innovative, rapid and dependable reverse-phase Ultra Performance Liquid Chromatography method for the simultaneous quantification of the anticancer drugs Abiraterone and Niraparib in bulk and pharmaceutical formulations marketed under the brand name Akeega. By offering a precise and stability-indicating assay, this research addresses a critical need for efficient analytical methods to assess these two agents in combination, an area with limited prior exploration. This novel approach not only fills a significant gap in the quantification of these compounds but also enhances analytical reliability for combined anticancer therapies, supporting broader research and quality control efforts.

Method: The method was optimized for isocratic elution on a C18 HSS column (2.1 mm × 100 mm, 1.8 µm) using a mobile phase composed of methanol and buffer 60:40v/v at a flow rate of 0.3 mL/min providing stable performance at room temperature. Detection was carried out with a UV detector set to 259 nm using a 10 µL sample injection volume and a total run time of five minutes.

Results: The retention times for Abiraterone and Niraparib were observed at 1.0333 and 3.4833 minutes, respectively, demonstrating excellent peak separation and resolution. The method showed strong linearity within concentration ranges of 12.5–75 µg/mL for Abiraterone and 2.5–15 µg/mL for Niraparib with calibration curve regression equations of Y = 9668x - 3531 (R^2 = 0.999) for Abiraterone and Y = 9632x + 1803 (R^2 = 0.999) for Niraparib. The % RSD values indicating precision were below 2 at 0.239 and 0.265. The method yielded percentage mean recoveries of 99.4-99.7% for Abiraterone and 99.5-99.8% for Niraparib with % RSD values ranging from 0.1-0.2 and 0.1-0.3 respectively. Rigorous forced degradation tests, including acidic, alkaline, oxidative, photolytic, and thermal conditions, confirmed the method's effectiveness as a stability-indicating assay.

Conclusion: Following validation in alignment with International Council for Harmonization (ICH) guidelines the method was found to be linear, specific, accurate, robust, time-efficient and suitable for quality control and process monitoring in the bulk manufacturing of these drugs. This validated method offers a valuable tool for ensuring the quality and stability of Abiraterone and Niraparib supporting their development and regulatory compliance.

Keywords: Reverse phase UPLC, Abiraterone, Niraparib, Stability indicating method, Method development, Method validation, forced degradation studies

1. INTRODUCTION

The combination of anticancer drugs Abiraterone and Niraparib was approved by Food and Drug Administration in the year 2023 with brand name Akeega is used to treat prostate cancer and other types of cancers. Metastatic castration-resistant prostate cancer and metastatic high-risk castration-sensitive prostate cancer can be treated with an antiandrogen called abiraterone [1]. Abiraterone suppresses (CYP17), 17α-hydroxylase/C17,20-lyase a crucial enzyme

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in the production of androgen. It is predominantly seen in testicular, adrenal, and prostatic malignancies. Inhibiting CYP17 can also lead to enhanced mineralocorticoid synthesis by the adrenals. [2]. chemical name for abiraterone is "(3S,8R,9S,10R,13S,14S)-10,13-dimethyl-17-pyridin-3-yl2,3,4,7,8,9,11,12,14,15-decahydro-1Hcyclopenta[a]phenanthren-3-ol". Its molecular formula is $C_{24}H_{31}NO$ with a molecular weight of 349.509 g/mol. It appears as a white or off-white solid and is soluble in ethanol, DMSO, and dimethyl formamide [3]. Niraparib a poly-ADP ribose polymerase inhibitor is employed for the management of recurrent peritoneal carcinoma, fallopian tube, or persistent ovarian epithelial cancer which responds to chemotherapy with platinum [4]. Niraparib substantially and specifically blocks the polyADP-ribose polymerase (PARP) enzymes PARP-1 and 2[5,6]. PARPs play a crucial role in DNA repair by detecting and repairing intracellular DNA damage, including single-strand breaks (SSBs) and double-strand breaks. Its IUPAC name "2-[4-[(3S)-piperidin-3-yl] phenyl] indazole-7-carboxamide"[7]. Its chemical formula and weight is $C_{19}H_{20}N_4O$ and 320.396 gm/mole. It appears as an off-white to white crystalline solid, soluble in ethanol and DMSO but insoluble in water. Chemical structures of Abiraterone and Niraparib is displayed in (Figure 1[8] and 2[9]).

A comprehensive review of the literature revealed that there were not many analytical techniques described for the UHPLC, HPLC, or UPLC assay of abiraterone alone or in combination with other anti-tumor agents [10–17]. How ever, for the simultaneous estimation of abiraterone and niraparib, no stability indicating RP-UPLC technique was reported [18–25]. Therefore, a UPLC stability-indicating method must be created to be concurrent assessment of the two medicines in their formulation and bulk. A RP-UPLC stability-indicating approach for the concurrent assessment of abiraterone and niraparib was attempted to be developed in this work.

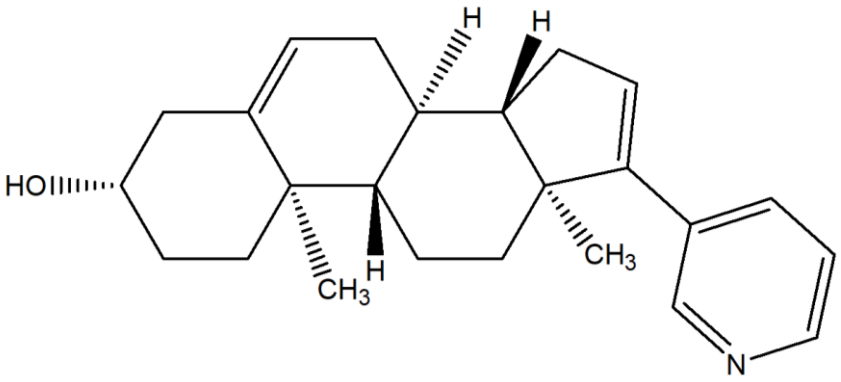

Figure 1: Chemical Structure of Abiraterone

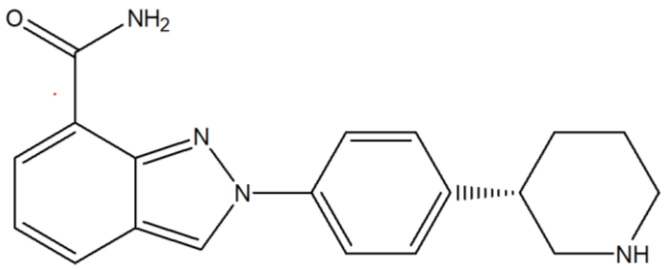

Figure 2: Chemical Structure of Niraparib

2. MATERIALS AND METHODS

Drug and dosage form

The drug samples of Abiraterone and Niraparib was obtained from Dr. Reddy's Laboratories, Hyderabad with 99.15% purity. Brand name akeega Tablets having 500 mg of Abiraterone and 100 mg Niraparib was purchased from local pharmacy. The chemicals utilized in the investigation such as glacial acetic acidand sodium acetate trihydrate were acquired from Merck chemicals in Mumbai. Merck Ltd. supplied HPLC-grade chemicals such as methanol and acetonitrile.

Instrumentation

Waters aquity UPLC System employed with Binary pump, autoinjector and UV detector. The data was acquired from Waters Empower software. Electronic weighing balance (Denver-SI-234 Bohemia) for weighing of all materials.pH meter (Systronics-Sr No S 1326 INDIA) was used to adjust pH of buffer solution. Ultrasonicator with (1.5L Capacity, GT Sonic INDIA) was used to sonicate the solutions. Vacuum Filtration (Borosilicate Vacuum Filtration Kit) was used to filter the solutions

Chromatographic conditions

The mobile phase consists of a accurately measured methanol and sodium acetate buffer 60:40 ratio. Mix the solvents completely using ultrasonic bath sonicator. Chromatographic separation was performed in isocratic elution using an HSS C18 (2.1 mm x 100 mm, 1.8 micron) column at room temperature at a flow rate of 0.3 mL/min. The sample injection volume was 10µL.

Preparation of solutions

Preparation of pH 4.8 sodium acetate buffer solution

13.608 g of sodium acetate trihydrate was dissolved in 800 mL of distilled water. Add approximately 3.2 mL of glacial acetic acid and mix the content for 2 min. pH of the solution was tune to 4.8 using glacial acetic acid and makes the solution up to the calibration mark in the flask.

Preparation of Mobile phase

Mix Methanol and buffer in the ratio of 60:40.

Diluent: Methanol is used as diluent.

Preparation of standard solution

Abiraterone and Niraparib, each weighing 50 mg, were separately dissolved in clean, dried 50 mL volumetric flasks. 50 mL of diluent was added to each flask and the mixtures were sonicated for two min to ensure complete dissolution of the analytes. The solutions were then filtered using a 0.2 µm membrane filter into separate clean, dry flasks, with the same solvent used to adjust the final volume. Separately, solutions of standard niraparib and abiraterone were composed at a concentration of 1000 µg/mL. From these solutions, 1 mL was shifted into a dry 10 mL volumetric flask and the volume was adjusted to 10 mL with diluent to achieve a concentration of 100 µg/mL for both abiraterone and niraparib. A 1 mL aliquot of this solution was further diluted in a 10 mL dry volumetric flask to obtain a final concentration of 10 µg/mL of niraparib which was used for the study.

Preparation of Sample

After ten tablets were taken, they were finely powdered. 50 mL of solvent were added to a 50 mL flask containing a precisely weighed tablet powder equivalent to 50 mg of abiraterone. A 50 mL volumetric flask was thoroughly cleaned and dried before the solution was filtered through a 0.2µ membrane filter after being sonicated for two minutes using an ultrasonic bath sonicator. With the same diluent and the formulation stock solution containing 1000 µg/mL of abiraterone, the final volume was adjusted to the required level. To get 50 µg/mL of abiraterone, 1 ml of the aforementioned solution is diluted with 20 ml of solvent. 10 µg/mL of Niraparib is present in the formulation solution based on the dosage in the formulation of both medicines. Abiraterone and Niraparib in the formulation sample were quantified using this formulation solution.

Method Development

An attempt was made in the proposed work to create and evaluate a novel, quick stability indicating RP-UPLC method for the estimation of two drugs simultaneously in bulk and dosage form.

Selection of wavelength

A spectrophotometer was used to establish the maximum absorption wavelength for the detection of niraparib and abiraterone. Each standard solution of niraparib and abiraterone was scanned in the 200–400 nm range. The iso-absorption wavelength of Abiraterone and Niraparib was fixed at 259nm was shown in (Figure 3) as detection wavelength for further study.

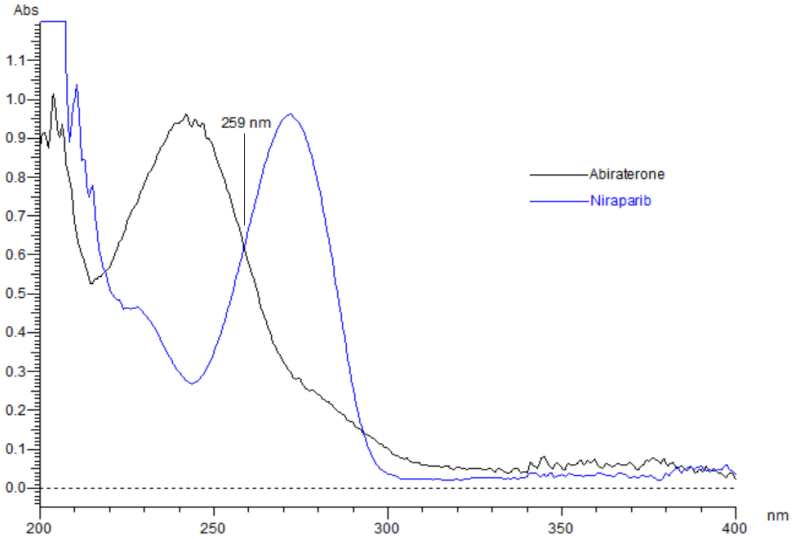

Figure 3: Overlay spectrum of Abiraterone and Niraparib

Optimization of chromatographic conditions

Attempts were made to develop an UPLC method for the selected combination on an isocratic mode; by using Methanol, acetonitrile and different buffers of different compositions were made to optimize the method. In conclusion, satisfactory separation of peaks with good resolution was attained on C18HSS (2.1mmx100 mm,1.8µm) column. Mobile phase consists of Methanol: buffer in 60:40 (v/v) with 0.3 mL/min flow rate at a wavelength of 259nm. The optimized chromatogram and conditions are shown in (Figure 4 and Table 1).

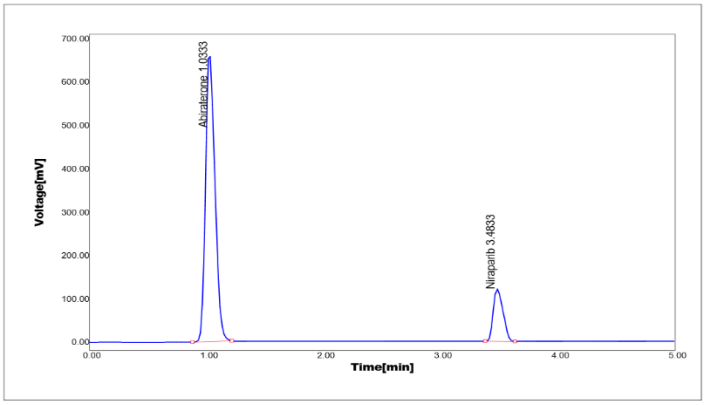

Figure 4: Optimized Chromatogram of Abiraterone and Niraparib

Table 1: Optimized Chromatographic conditions

Parameters	Condition
Column	C18HSS (2.1mm x100 mm, 1.8µm)
Mobile phase	Methanol: Buffer 60:40 (v/v)
Flow rate	0.3 mL/min
Temperature of Column	Ambient
Temperature of Sample	Ambient
Detection wavelength	259 nm
Volume of Injection	10 µL
Pump mode	Isocratic
Run Time	5 minutes
Retention time	Abiraterone -1.0333 minutes Niraparib-3.4833 minutes

Method Validation

The analytical method was validated for criteria such as system suitability, specificity, linearity, precision, accuracy, robustness, Limit of detection and Limit of Quantification forced degradation studies in accordance with ICH Q2 (R1).

1. System suitability

System performance was assessed using system suitability metrics. In order to ensure system appropriateness, 10µl of a standard solution containing Abiraterone (50 µg/mL) and Niraparib (10 µg/mL) was injected into the UPLC system six times.

2. Specificity

The ability to precisely measure an analyte of interest in the presence of additional components in a sample matrix is evaluated using the specificity parameter during technique validation.

3. Linearity

Preparation of sample solutions for Linearity

Standard calibration curve were prepared with the injection of working standard solutions at six concentration levels (Abiraterone 12.5, 25, 37.5, 50, 62.5, and 75µg/mL) and (Niraparib 2.5, 5, 7.5, 10,12.5 and 15µg/mL). The peak area responses at each concentration level for all the drugs were determined using the described chromatographic conditions.

4. Precision

The standard which contained Abiraterone(50µg/mL) and Niraparib(10µg/mL) was examined six times in a single day for intraday precision and three times over the

course of two days for interday precision.

5. Accuracy

A 50%, 100%, and 150% spiked version of the approach was used to test accuracy. The enhanced approach examined the augmented level solution of niraparib and abiraterone.

6. Robustness

The robustness investigation was carried out by slightly adjusting physical factors such as the detection wavelength of ±5 nm, pH of the mobile phase ±1, and the composition of the mobile phase ±5 ml.

7. Limit of Detection and Limit of Quantification

A study was carried out to determine the LOD and LOQ for abiraterone and niraparib. In accordance with the test protocol, a series of extremely diluted LOD and LOQ solutions were made and triple-injected into the UPLC system.

Forced Degradation Studies

The following stress conditions were used in stress research to show how well the sample's deterioration could be separated from its primary analyte peaks. All of the stressed samples were injected twice into the UPLC system under optimal chromatographic circumstances after being diluted to the necessary concentration using diluents. The chromatograms were then recorded and assessed for the assay and degradation percentages. Abiraterone and Niraparib's percentage degradation was computed.

Sample preparation for degradation: To 5 ml of stock solution of abiraterone and 10ml of stock solution of niraparib were mixed with 10 ml of 0.1N HCl,0.1N NaOH,3% Peroxide solution, oven at 60^0C, UV light 254 nm. The resultant solutions were diluted to obtain a concentration of (50 µg/mL & 10µg/mL) and 10µL were injected into UPLC system and the chromatogram were recorded to assess the stability of sample.

3. RESULTS

1. System suitability

The findings for both medications were listed in (Table 2) and the standard chromatogram is displayed in (Figure 5) indicating that the system suitability parameters were within the limit.

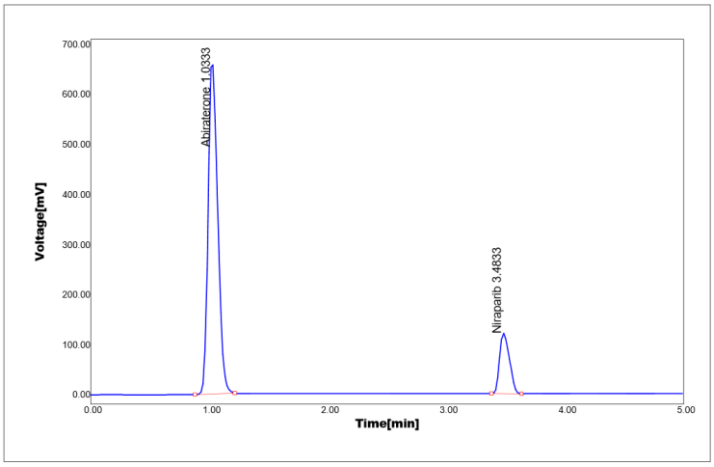

Figure 5: Chromatogram of Standard-Retention time of abiraterone at 50 µg/mL is 1.0333 and Retention time of niraparib at 10 µg/mL is 3.4833

Table 2: System suitability parameters

Parameter	Abiraterone	Niraparib
Theoretical plates	5237	9458
Tailing factor	0.98	1.03
Resolution	-	16.25
Retention Time	1.0333	3.4833

2. Specificity

It was performed by injecting blank (mobile phase only), Abiraterone, Niraparib individually and combined solutions. The chromatograms are displayed in (Figures 5,6,7,8).

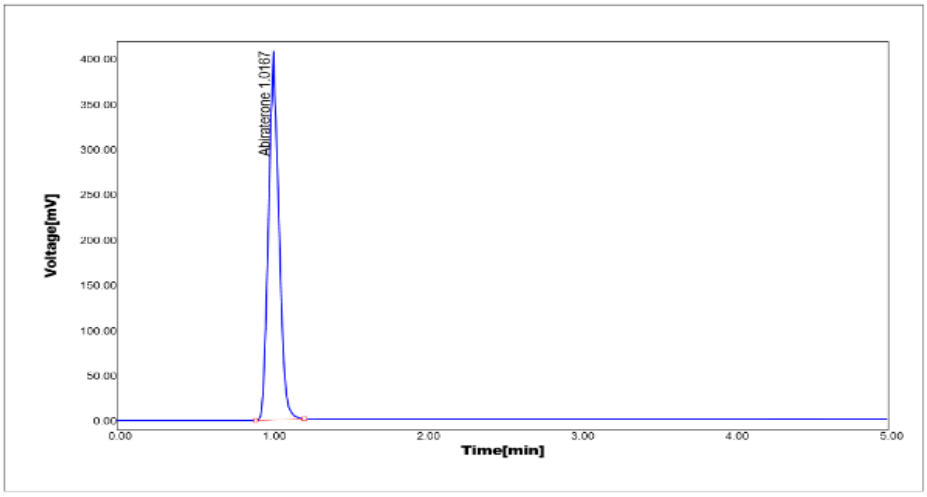

Figure 6: Chromatogram of individual abiraterone-showing a well-defined peak at 1.0167 minutes corresponding to abiraterone's retention time under optimized chromatographic conditions

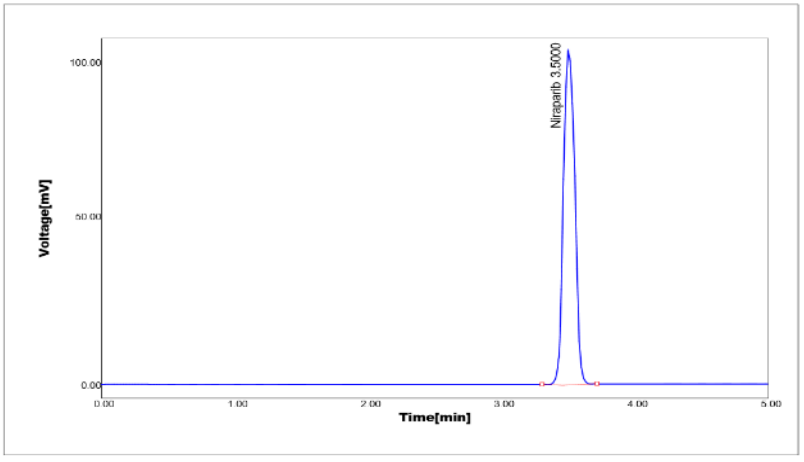

Figure 7: Chromatogram of Individual Niraparib-Retention time at 3.5000

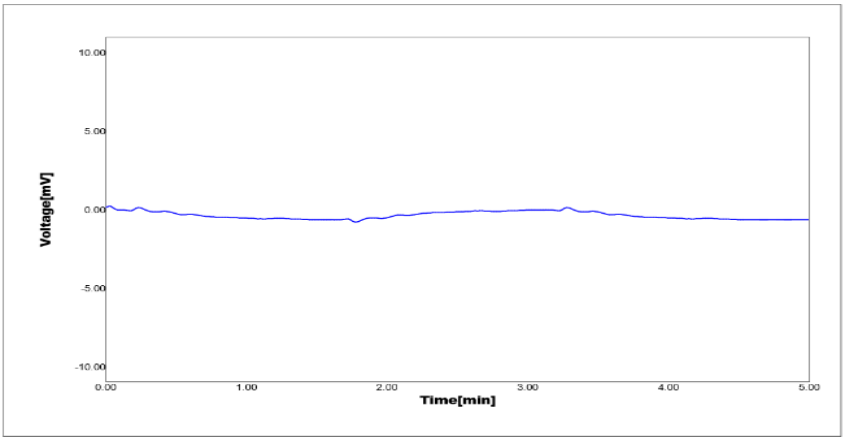

Figure 8: Chromatogram of Blank – Run with only mobile phase Methanol and buffer in the ratio of 60:40v/v.

3. Linearity

The calibration curve for Abiraterone (12.5-75 µg/mL) and Niraparib (2.5-15 µg/mL) were found to be linear. The regression equations for Abiraterone and Niraparib were Y=9668x-3531 (R2-0.999) and Y=9632.x+1803 (R2-0.999) respectively which were shown in the (Table 3).

Table 3: Linearity results for Abiraterone and Niraparib

S.No.	Abiraterone		Niraparib	
	Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
1	12.5	117384.3	2.5	25417.8
2	25	239172.3	5	50882.3
3	37.5	356702.1	7.5	73934.2
4	50	486243.4	10	98812.7
5	62.5	590889.6	12.5	119847.3
6	75	726459.4	15	147632.2
Slope	9668.7		9632.5	
Intercept	3531.7		1803.2	
Correlation Coefficient	0.999		0.999	

4. Precision

The response of peak area and the percentage RSD of Abiraterone and Niraparib was tabulated. The %RSD is less than 2 in both the precision studies for Abiraterone and Niraparib were regarded satisfactory and is shown in (Tables 4 and 5).

Table 4: Results of Intraday Precision

S.No.	Abiraterone		Niraparib	
	Concentration (µg/mL)	**Average Area(n)**	**Concentration (µg/mL)**	**Average Area(n)**
1	50	485659.9	10	98986.6
2	50	485076.4	10	98783.1
3	50	483034.2	10	98239.6
4	50	486000.3	10	98476.7
5	50	484736.1	10	98753.4
6	50	486194.8	10	98625.3
Mean	485117		98644.12	
Std dev	1159.431		260.9867	
% RSD	0.239		0.265	

Table 5: Results of Interday Precison

S.No.	Abiraterone		Niraparib	
	Day 1	**Day 2**	**Day 1**	**Day 2**
1	487556.3	485125.4	98747.5	98466.9
2	482499.3	484541.5	98684.2	98407.6
3	483569.1	482936.9	98575.5	98249.5
Mean	**484541.6**	**484201.3**	**98669.07**	**98374.67**
Std dev	**2665.067**	**1133.226**	**86.99289**	**112.3795**
% RSD	**0.550**	**0.234**	**0.088**	**0.114**

5. Accuracy

The percentage recovery range of 98-102 was deemed satisfactory. (Tables 6 and 7) summaries the accuracy results.

Table 6: Accuracy results of Abiraterone

S.No	Recovery Level	Concentration (µg/mL)			Average Area(n)	Amount found (µg/mL)	% Recovery	% Mean Recovery &% RSD
		Target	**Spiked**	**Final**				
1	50 %	25	12.5	37.5	356595.1	37.49	99.97	99.68 0.277
2		25	12.5	37.5	354633.2	37.28	99.42	
3		25	12.5	37.5	355489.3	37.37	99.66	
4	100 %	25	25	50	485611.3	49.94	99.87	99.76 0.110
5		25	25	50	485076.4	49.88	99.76	
6		25	25	50	484541.5	49.82	99.65	
7	150 %	25	37.5	62.5	586339.8	62.02	99.23	99.48 0.273
8		25	37.5	62.5	587639.7	62.16	99.45	
9		25	37.5	62.5	589530.6	62.36	99.77	

Table 7: Accuracy Results of Niraparib

S.No	Recovery Level	Concentration (µg/mL)			Average Area (n)	Amount Found µg/mL	% Recovery	% Mean Recovery & % RSD
		Target	Spiked	Final				
1	50 %	5	2.5	7.5	73675.4	7.474	99.65	99.55 0.114
2		5	2.5	7.5	73631.1	7.469	99.59	
3		5	2.5	7.5	73512.8	7.457	99.43	
4	100 %	5	5	10	98427.3	9.961	99.61	99.76 0.142
5		5	5	10	98605.2	9.979	99.79	
6		5	5	10	98704	9.989	99.89	
7	150 %	5	7.5	12.5	119883.3	12.504	100.03	99.82 0.325
8		5	7.5	12.5	119188.1	12.431	99.45	
9		5	7.5	12.5	119835.3	12.499	99.99	

6. Robustness

The solution of standard produced according to the test protocol was put into the UPLC instrument. (Tables 8 and 9) show the results of the robust study.

Table 8: Robustness results of Abiraterone

Parameter	Optimized conditions	Used condition	Peak area	Retention time	Tailing factor	Theoretical plates
Mobile phase Composition	Methanol: buffer (60:40 v/v)	Methanol: buffer (65:35 v/v)	484590.2	1.0167	0.99	5264
		Methanol: buffer (55:45 v/v)	485951.7	1.0333	0.98	5301
pH of mobile phase	4.8	pH changed as 4.7	485319.5	1.0000	0.99	5142
		pH changed as 4.9	484541.5	1.0500	0.98	5091
Detector wavelength	259	Detector wavelength 264nm	484249.8	1.0000	0.99	5145
		Detector wavelength 254nm	483471.8	1.0333	0.99	5388

Table 9: Robustness results of Niraparib

Parameter	Optimized conditions	Used condition	Peak area	Retention time	Tailing factor	Theoretical plates
Mobile phase Composition	Methanol: buffer (60:40 v/v)	Methanol: buffer (65:35 v/v)	98763.3	3.6833	1.06	9571
		Methanol: buffer (55:45 v/v)	98506.4	3.4333	1.04	9385
pH of mobile phase	4.8	pH changed as 4.7	98802.8	3.4667	1.07	9507
		pH changed as 4.9	98585.4	3.5000	1.05	9324
Detector wavelength	259	Detector wavelength 264nm	98466.9	3.4833	1.03	9313
		Detector wavelength 254nm	98140.8	3.7167	1.05	9519

7. Limit of Detection and Limit of Quantification

Signal to noise ratio was used to determine the limit of detection and limit of quantification. The sensitivity results are presented in (Table 10).

Table 10: Results of LOD and LOQ

S No	Analyte	Sensitivity	
		LOD	LOQ
1	Abiraterone	0.25 µg/mL	0.825 µg/mL
2	Niraparib	0.05µg/mL	0.165 µg/mL

Forced Degradation Studies

(Tables 11 and 12) provide the degradation data for abiraterone and niraparib.

Table 11: Degradation studies of Abiraterone

Stress condition	Area value	% Degradation	% Assay
Acid	485708.5	0.110	99.89
Base	486194.8	0.010	99.99
Peroxide	484006.7	0.460	99.54
Thermal	482936.9	0.680	99.32
UV light	485659.9	0.120	99.88

Table 12: Degradation studies of Niraparib

Stress condition	Area value	% Degradation	% Assay
Acid	98536.3	0.280	99.72
Base	98160.5	0.660	99.34
Peroxide	98140.8	0.680	99.32
Thermal	98802.8	0.010	99.99
UV light	98377.9	0.440	99.56

Assay

The commercial tablet (AKEEGA) was examined individually by introducing 10µL of solutions of standard and sample into the UPLC machine and then recording chromatograms. By comparing standard and sample peak areas, the quantity of the medication included in marketed tablets was determined. (Table 13) displays the assay results whereas (Figure 11) depicts the chromatogram.

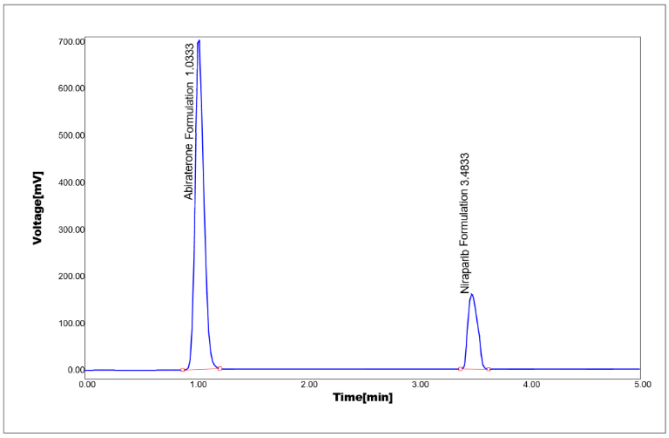

Figure 11: Chromatogram of Marketed Formulation

Table 13 : Results of Assay

S.No	Drug	Brand Name	Dosage	Area value	Concentration prepared(µg/ml)	Concentration found (µg/ml)	% Assay
1	Abiraterone	AKEEGA	500 mg	485453.4	50	49.919	99.84
2	Niraparib		100 mg	98445.1	10	9.96	99.63

4. DISCUSSION

The ultra-performance liquid chromatography (UPLC) method was optimized and validated as a stability-indicating approach for the simultaneous estimation of abiraterone and niraparib in bulk and dosage forms. The analysis was performed using isocratic elution on a C18 HSS column (2.1 mm × 100 mm, 1.8 µm) with a flow rate of 0.3 mL/min at ambient temperature and an injection volume of 10 µL over a 5-minute run time. The mobile phase consisted of methanol and buffer (60:40), and detection was carried out using a UV detector at 259 nm. The retention times were 1.0333 min for abiraterone and

3.4833 min for niraparib. The results of the optimized chromatographic conditions are presented in Table 1.

System suitability parameters, including tailing factor and theoretical plates, were within acceptable limits when standard solutions of both drugs were injected six times. Typical chromatograms for sample formulations, individual drug solutions, and blanks showed no interfering peaks, demonstrating the method's specificity. Calibration curves were linear in the ranges of 12.5–75 µg/mL for abiraterone and 2.5–15 µg/mL for niraparib, with regression equations of $Y = 9668x - 3531$ ($R^2 = 0.999$) and $Y = 9632x + 1803$ ($R^2 = 0.999$), respectively, indicating excellent correlation between peak area and concentration.

Precision was confirmed by six injections of 50 µg/mL abiraterone and 10 µg/mL niraparib, with %RSD values of 0.239 and 0.265, respectively, demonstrating high reproducibility. Accuracy was validated through recovery studies, with mean recoveries and %RSD of 99.4–99.7% (0.1–0.2) for abiraterone and 99.5–99.8% (0.1–0.3) for niraparib. Robustness was assessed by varying mobile phase composition (±5 mL), pH (±1), and wavelength (±5 nm), with no significant effect on results.

The limits of detection (LOD) and quantification (LOQ) were 0.25 and 0.825 µg/mL for abiraterone and 0.05 and 0.165 µg/mL for niraparib, indicating high method sensitivity. Stress studies under acidic, alkaline, oxidative, thermal, and photolytic conditions confirmed the method's stability-indicating capability, with degradation clearly distinguishable from the principal analyte peaks. Stressed samples were appropriately diluted and injected twice, with percentage degradation and assay values within acceptable limits.

The validated method was successfully applied to commercial tablet formulations, yielding assay results of 99.84% for abiraterone and 99.63% for niraparib.

5. CONCLUSION

The present study describes a novel UPLC stability-indicating method for the determination of abiraterone and niraparib in bulk and tablet formulations. The developed method offers several advantages: it eliminates the need for time-consuming extraction steps, simplifies solution preparation, and allows chromatogram recording within five minutes. Consequently, the method is fast, simple, sensitive, accurate, and reliable. It has been successfully applied in research laboratories, industrial quality control departments, and accredited testing facilities for routine analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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تطوير واعتماد طريقة جديدة شاملة لتحريض الاستقرار لتقييم أبيراتيرون ونيرباريب في تركيبة صيدلانية وتجميلية باستخدام تقنية كروماتوغرافيا السائل فائقة الأداء (UPLC)

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ملخص

الخلفية :تهدف هذه الدراسة إلى تطوير واعتماد طريقة مبتكرة وسريعة وموثوقة للكروماتوغرافيا السائلة فائقة الأداء في الطور العكسي، وذلك للتقدير الكمي المتزامن لعقاري أبيراتيرون ونيراباريب المضادين للسرطان، في تركيبات سائبة وصيدلانية تُسوّق تحت العلامة التجارية "أكيغا ."ومن خلال تقديم تحليل دقيق ومُؤشر على الثبات، يُلبي هذا البحث حاجةً ماسةً إلى أساليب تحليلية فعّالة لتقييم هذين العاملين معًا، وهو مجالٌ لم يُستكشف سابقًا إلا بشكل محدود .ولا يقتصر هذا النهج المبتكر على سد فجوة كبيرة في تقدير كميات هذه المركبات، بل يُعزز أيضًا موثوقية التحليلات لعلاجات السرطان المُركّبة، مما يدعم جهودًا بحثيةً أوسع نطاقًا ومراقبة جودة.

الطريقة :تم تحسين الطريقة لإجراء عملية إفلات متساوي الكثافة على عمود من الفولاذ عالي السرعة 2.1) C18 مم 100 × مم، 1.8 ميكرومتر (باستخدام طور متحرك مكون من الميثانول ومحلول منظم 60:40 فولت/فولت بمعدل تدفق 0.3 مل/دقيقة، مما يوفر أداءً مستقرًا في درجة حرارة الغرفة .تم الكشف باستخدام كاشف للأشعة فوق البنفسجية مضبوط على 259 نانومتر ، باستخدام حجم حقن عينة 10ميكرولتر ، ومدة تشغيل إجمالية خمس دقائق.

النتائج :لوحظت أزمنة بقاء أبيراتيرون ونيراباريب عند 1.0333 و 3.4833دقيقة على التوالي، مما يدل على فصلٍ ودقةٍ ممتازين للذروة . أظهرت الطريقة خطيةً قويةً ضمن نطاقات تركيز تتراوح بين 12.5 و 75ميكروغرام/مل لأبيراتيرون و 2.5و 15ميكروغرام/مل لنيراباريب، مع معادلات انحدار منحنى المعايرة ($Y = 9668x - 3531$ ($R^2 = 0.999$ لأبيراتيرون و ($Y = 9632x + 1803$ ($R^2 = 0.999$لنيراباريب . كانت قيم الانحراف المعياري النسبي (%RSD) التي تشير إلى الدقة أقل من 2 عند 0.239 و .0.265حققت الطريقة متوسط نسبة استرداد تراوحت بين 99.4% و 99.7%لأبيراتيرون و 99.5%و 99.8%لنيراباريب، مع قيم انحراف معياري نسبي (RSD) تراوحت بين 0.1% و 0.2%و 0.1%و 0.3%على التوالي .وقد أكدت اختبارات التحلل القسري الصارمة، التي شملت الظروف الحمضية والقلوية والأكسدة والتحلل الضوئي والحراري، فعالية الطريقة كاختبار مؤشر على الثبات.

الاستنتاج :بعد التحقق من صحة هذه الطريقة، بما يتماشى مع إرشادات المجلس الدولي للتنسيق (ICH)، وُجد أنها خطية، ومحددة، ودقيقة، ومتينة، وموفرة للوقت، ومناسبة لمراقبة الجودة ومراقبة العمليات في التصنيع بالجملة لهذه الأدوية .تُقدم هذه الطريقة المُعتمدة أداةً قيّمة لضمان جودة واستقرار أبيراتيرون ونيراباريب، مما يدعم تطويرهما وامتثالهما للأنظمة.

الكلمات الدالة: مرحلة عكسية من UPLC، أبيراتيرون، نيراباريب، طريقة الإشارة إلى الاستقرار، تطوير الطريقة، التحقق من صحة الطريقة، دراسات التحلل القسري.

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عمادة البحث العلمي

المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

تأسست المجلة الأردنية في العلوم الصيدلانية بقرار لجنة البحث العلمي/ وزارة التعليم العالي والبحث العلمي رقم 367/2/10 تاريخ 2007/1/11 بشأن إصدار "**المجلة الأردنية في العلوم الصيدلانية**" ضمن إصدارات المجلات الأردنية الوطنية، وهي مجلة علمية عالمية متخصصة ومحكمة، وتصدر بدعم من صندوق دعم البحث العلمي والجامعة الأردنية تعنى بنشر البحوث العلمية الأصيلة المقدمة إليها للنشر في كافة مجالات العلوم الصيدلانية والعلوم الأخرى المرتبطة بها. وتصدر عن عمادة البحث العلمي وضمان الجودة في الجامعة الأردنية باسم الجامعات الأردنية كافة، خدمة للمتخصصين والباحثين والمهتمين في هذه المجالات من داخل الأردن وخارجه. وهي مجلة تصدر أربع مرات في العام أعتبارا من 2021، ومواعيد صدورها (آذار وحزيران وأيلول وكانون أول) من كل عام.

وباسمي وباسم أعضاء هيئة التحرير نود أن نشكر الزملاء الذين أسهموا بإرسال أبحاثهم إلى مجلتنا وتمكنا من إخراج العدد الأول. ونأمل من جميع الزملاء بإرسال ملاحظاتهم الإيجابية إلينا لنتمكن من النهوض بمجلتكم بالشكل الذي يليق بها.

وهذه دعوة إلى كافة الزملاء لإرسال اسهاماتهم العلمية من الأبحاث الأصيلة إلى عنوان المجلة.

والله ولي التوفيق

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