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Jordan Journal of Pharmaceutical Sciences

Aims & Scopes:

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

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INTRODUCTION

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

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

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

Volume 16, 2023

Letter from the Editor-in-Chief

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

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

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

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

Best regards

Prof Ibrahim Alabbadi
Editor-in-Chief

Editorial Commentary

Artificial intelligence (AI) has made significant advancement in the field of pharmacy and, revolutionized various aspects of the industry. Among the most affected practices are drug discovery, design of dosage forms, poly-pharmacology and hospital pharmacy.

AI has proven to be invaluable in drug discovery, where it can help identify potential drug candidates from vast amounts of data. AI algorithms can analyze molecular structures, biological targets, predict drug-target interactions, propose novel compounds with desired properties, prioritize promising drug candidates for further development and analyze clinical trials results. Machine learning techniques can also accelerate the screening process by analyzing large databases of existing drugs and their effects, identifying patterns, and suggesting potential new therapeutic uses.

AI can assist in the design and optimization of dosage forms. By analyzing factors such as, drug properties, release profiles, leveraging patient-specific data, and identifying the most effective formulations. AI can also assist in predicting drug dissolution, absorption, and release patterns, enabling the development of more effective and efficient drug delivery systems. Machine learning techniques can optimize drug delivery systems, such as nanoparticles or microspheres, by considering parameters like drug release kinetics, stability, and target site specificity. AI can also help in personalized medicine by tailoring dosage forms to individual patients' needs, taking into account factors like age, weight, and genetic variations.

AI can help in identifying potential poly-pharmacological effects of drugs by analyzing large-scale molecular and biological data and studying the complex interactions between multiple drugs. By applying network analysis, AI algorithms can predict the interaction between drugs and various biological targets, identify potential drug combinations for synergistic effects or minimize adverse interactions. AI can aid in the design of multi-target drugs, enhancing their efficacy and reducing side effects.

Hospital Pharmacy: AI-powered systems can assist in medication management by analyzing patient data, drug interactions, and allergies to prevent medication errors, medication reconciliation, and dosage adjustments. AI can also optimize inventory management by predicting medication demand, expiration dates, and ensuring adequate stock levels.

Overall, AI has the potential to significantly affect the field of pharmacy. By leveraging its capabilities in data analysis, pattern recognition, and predictive modeling. However, it is important to note that while AI can provide valuable insights, it should always be used in conjunction with human expertise to ensure patient safety and ethical considerations are addressed.

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CONTENTS

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Investigation of Nootropic and Neuroprotective Activity of *Myristica malabarica* Bark Extracts on STZ induced Cognitive Impairment in Experimental Animals

Kolguri Jagadeeshwar[1], Subhakar Raju Rupaka[1], Rajasekhar Reddy Alavala[1,2], GSN Koteswara Rao[1,2], Umasankar Kulandaivelu[1*]

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ABSTRACT

The present study aims to assess the *Myristica malabarica* Bark (MM) extracts in the diabetes-induced cognitive impaired rat model for its nootropic and neuroprotective activity. Memory enhancing activity was evaluated by Y maze and Morris water maze test, respectively. Neuroprotective effects of MM bark extracts were assessed by measuring the acetylcholinesterase, lipid peroxide, superoxide dismutase (SOD), total nitric oxide (NO), catalase (CAT) and glutathione (GSH) levels in the brain of diabetic rats. The *Myristica malabarica* bark methyl alcohol extract (MMMA) (100 and 200 mg/kg) was observed to affect a significant improvement in spontaneous alteration ($P<0.01$, $P<0.001$) and transfer latency ($P< 0.01$ $P<0.001$) in retention trials on Y maze and Morris water maze test respectively. However, a significant reduction in acetylcholinesterase activity ($P<0.001$), lipid peroxide ($P<0.001$), total NO ($P< 0.001$) and a substantial increase in SOD, CAT and GSH ($P<0.001$) levels was observed in animals treated with MMMA (200 mg/kg) related to the diabetic control group. The current results indicate that *Myristica malabarica* extracts were defending the cognitive decline in diabetes condition and which requires some additional studies to clarify its mode of action.

Keywords: *Myristica malabarica*; Nootropic; Neuroprotective; Cognitive decline.

INTRODUCTION:

Diabetes mellitus (DM) is a clinical condition with a set of symptoms, considered by hyperglycemia owing to a complete or comparative lack of insulin or non-responsiveness of tissues to insulin which affects at least 382 million people worldwide [1]. DM is regarded as a cause of high mortality and morbidity rate due to many physiological complications. Cognitive dysfunction has been considered the greatest prevalent and significant one, especially in older people with type 2 diabetes mellitus (T2DM) [2].

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Alzheimer's disease (AD), a chronic neurodegenerative disease, is the utmost common form of dementia regarded as a warning sign of temporary memory loss i.e., difficulty in remembering recent events, this happens to owe to the loss of intelligence cells [3]. Correlation between Alzheimer's disease and diabetes have been well established. Many clinical and epidemiological studies revealed that the pathophysiological features of diabetes and neuropathic disease are comparable to both, which share complex and connected mechanisms, including insulin resistance, inflammation, and oxidative stress [4].

Besides, the impairment of insulin signaling in the brain may injure the capability of neurons to self-repair and could enhance the development of neurodegenerative disorders [5]. Many preclinical studies, both type-1 and type-2 diabetic models, used to cause severe memory

deficits [6]. The augmented oxidative stress in diabetes produces oxidative damage in many regions of the rat brain including hippocampus.

Plants show a principal role in discovering new therapeutic agents [7] and have been considered as sources of biologically active substances including antioxidants and hypoglycemic and neuroprotective agents [8, 9]. *Myristica malabarica* Lam (*Myristicaceae*) is widely distributed in the Western Ghats Forest region and is commonly called Malabar nutmeg or Kaatuhjathi. The plant was known to be used for antioxidant, antidiabetic, anti-ulcer, anti-inflammatory, analgesic, sedative, hypnotic, and antimicrobial actions [10]. The current study intended to assess the effectiveness in animals with *Myristica malabarica* (**MM**) on various cognitive and oxidative changes in STZ induced young diabetic rats having severe hyperglycemia (FBS ≥ 250 mg/dl).

MATERIAL AND METHODS:

Collection of Plant Materials

Myristica malabarica (**MM**) bark was collected from the tree in December 2018 from the Thiruvananthapuram, Kerala, India.

Chemicals

Piracetam (Alkem Laboratories Ltd), Metformin (Cipla Pharmaceuticals), Diagnostic Kits (Bio Lab, India), and Streptozotocin were bought from Sigma Aldrich, USA. Other chemicals used in the study were of analytical grade.

Experimental Animals

For this study the strains of wistar rats (150±50 gm) of both gender were selected and procured from Mahaveer enterprises, Hyderabad and acclimated to the fixed research laboratory temperature, needed humidity and with 12 h light/dark conditions set for one week. The animals remained fed through a consistent pellet diet and water *ad libitum*.

Preparation of MM Bark extracts

Soxhlet extractor was used for the extraction of **MM** bark for 72 hrs at the temperature below the boiling point of the solvent *via* increasing polarity order petroleum ether (**PE**), ethyl acetate (**EA**) and methanol (**MA**). Whatman filter paper (No.1) was used to filter the extract and later concentrated under vacuum. Finally, dried at 45°C and the dried extracts were remained preserved in a disinfected container and frozen till usage.

Phytochemical Investigation

Every plant extract remained vaporized to the residue and dilute hydrochloric acid was added to it. Subsequently mixed, dissolved and then filtered. The filtrate used for performing the identification tests for various phytochemical constituents [11].

Acute toxicity study

The acute toxicity test was performed as per the OECD guidelines No. 423. In each step, three animals were employed. The dosage range was chosen consisting of the four fixed-dose levels, *i.e*., 5, 50, 300, and 2000 mg/kg body weight *p.o* [12].

Diabetes Induction

After acclimatization all rats were kept in overnight fasting condition and randomly divided in to ten groups, each group contain six rats. For induction of diabetes in the experimental animals, 55 mg/kg of streptozotocin (STZ) was given *i.p* to the animals. For confirmation of diabetes, estimated the glucose level in the next 48 hours of STZ injection under light anesthesia. The glucose levels was evaluated by the GOD-POD method and the animals were observed to possess more than 250 mg/dl of blood glucose reflected as diabetic and recommended for additional investigation [13, 14].

Y maze Test

The treatment protocol was illustrated in Table 1. The restrained Y maze study was performed for prompt memories, which provides the complete alteration in behaviour. Animals to be situated on the end of any arm and recognized to way easily over the maze. The period limit continued steady to 8 mins so, every period ended 8 mins later. Limb admittance was considered when the back legs of the rat stayed totally inside the arm. Natural

alteration behaviour was perfect as entrance into wholly three arms on consecutive selections. After acclimatization all rats were kept in overnight fasting condition and randomly divided into ten groups, each group contain six rats. After recording initial reaction time, treatment with standard drugs (metformin and piracetam), PEMM (100 and 200 mg/kg), EAMM (100 and 200 mg/kg) and MAMM (100 and 200 mg/kg) was given to each rat. The each rat was kept in Y maze in order to record percentage spontaneous variations on day 71 & 75 [15].

Table 1: Protocol for evaluation of memory enhancing activity by Y maze test of MM extracts using rats.

Group	Status	Treatment
I	Normal Control (NC)	0.1% Sodium CMC
II	Disease Control (DC)	0.1% Sodium CMC+55 mg/kg Streptozotocin
III	Diabetes+Metformin	0.1% Sodium CMC+10 mg/kg Metformin
IV	Diabetes+Piracetam	0.1% Sodium CMC+5 mg/kg Piracetam
V	Diabetes+PEMM	0.1% Sodium CMC+100 mg/kg extract of PEMM
VI	Diabetes+PEMM	0.1% Sodium CMC+200 mg/kg extract of PEMM
VII	Diabetes+EAMM	0.1% Sodium CMC+100 mg/kg extract of EAMM
VIII	Diabetes+EAMM	0.1% Sodium CMC+200 mg/kg extract of EAMM
IX	Diabetes+MAMM	0.1% Sodium CMC+100 mg/kg extract of MAMM
X	Diabetes+MAMM	0.1% Sodium CMC+200 mg/kg extract of MAMM

PEMM=Petroleum Ether extract of Myristica malabarica. EAMM=Ethyl Acetate extract of Myristica malabarica. MAMM=Methyl alcohol extract of Myristica malabarica

Morris Water Maze Test

The treatment protocol was illustrated in Table 2. On day one rats were educated to swim for 60 sec in the non-existence of the stage. Throughout four consecutive days, rats remained assumed the probationary session through the stage. If rat locates the stage, allowed remaining continuously it intended for 10 sec. The rat not finds, placed again for same time and now detached on platform. After acclimatization all rats were kept in overnight fasting condition and randomly divided into ten groups, each group contain six rats. After recording initial reaction time, treatment with standard drugs (metformin and piracetam), PEMM (100 and 200 mg/kg), EAMM (100 and 200 mg/kg) and MAMM (100 and 200 mg/kg) was given to each rat and now rats stayed separately exposed to investigation test session the stage remained detached as of the pool and might swim for 120 sec to examine aimed at it. On day 71, animals were tested for latency time was determined [16].

Table 2: Protocol for evaluation of memory enhancing activity by Morris water maze test of MM extracts using rats.

Group	Status	Treatment
I	Normal Control (NC)	0.1% Sodium CMC
II	Disease Control (DC)	0.1% Sodium CMC+55 mg/kg Streptozotocin
III	Diabetes+Metformin	0.1% Sodium CMC+10mg/kg Metformin
IV	Diabetes+Piracetam	0.1% Sodium CMC+5mg/kg Piracetam
V	Diabetes+PEMM	0.1% Sodium CMC+100mg/kg extract of PEMM

Group	Status	Treatment
VI	Diabetes+PEMM	0.1% Sodium CMC+200mg/ kg extract of PEMM
VII	Diabetes+EAMM	0.1% Sodium CMC+100mg/kg extract of EAMM
VIII	Diabetes+EAMM	0.1% Sodium CMC+200mg/kg extract of EAMM
IX	Diabetes+MAMM	0.1% Sodium CMC+100mg/kg extract of MAMM
X	Diabetes+MAMM	0.1% Sodium CMC+200mg/kg extract of MAMM

Neurotoxicity Studies

After treatment schedule all group animals were forewent by cervical dislocation and brain was removed and weighed. Total brain was washed through ice cold saline and make uniform by take 20 mg of the tissue per ml in chilled phosphate buffer (pH 7.4). The homogenates were centrifuged at 800 rpm for 5 mins at 4°C to distinct the nuclear fragments. The obtained supernatant was centrifuged at 1050 rpm for 20 minutes at 4°C to get the supernatant. Such attained supernatant was then used for neurochemical estimation.

Estimation of Acetylcholinesterase (AChE)

AchE estimated by method of Ellman's named later George Ellman was used [17]. A total of 0.4 ml supernatant was added to 2.6 ml of phosphate buffer (0.1 mol/L, pH 8) and 100 µL of 5, 5′-dithiobis-(2-nitrobenzoic acid), then estimated absorbance by a spectrophotometer at 412 nm. The 20 µL of substrate mixed with acetylthiocholine-iodide and recorded the changes in absorbance for a period of 10 minutes at intervals of 2 minutes. Alteration in the absorbance per minute was measured and acetylcholinesterase activity was expressed as µM/l/min/gm of tissue [17].

TBARS Assay

Lipid peroxidation property of plant extracts evaluated as per the method of Wills et al [18]. Formation of MDA is crucial for thiobarbituric acid reactive substances (TBARS) levels, and it is stated in MDA/mg of protein.

Total nitric oxide levels

The 500 µl of Greiss reagent added to 100 µl of supernatant liquid then absorbance was estimated at 546 nm. The amount of nitrite was measured by using a standard curve for sodium nitrite and it is expressed as ng/mg of protein [19].

Superoxide dismutase (SOD) levels

SOD levels were estimated as per the method of Kono and for this supernatant (100 µl), sodium carbonate (1ml), of nitroblutetrazolin (0.4 ml) and ethylene diamine tetra acetic acid (0.2 ml) was added, later the absorbance was estimated at 560 nm and expressed in µg/mg of protein [20].

Catalase (CAT) levels

CAT amount in entire treatment groups estimated by the method of Claiborne et al [21]. A supernatant of 100 µl added with 1.9 ml of phosphate buffer measure the absorbance at 240 nm and it is specified as µg/mg of protein.

Glutathione (GSH) levels

In total treatment groups as GSH levels were assessed as per the method of Jollow et al [22]. The GSH levels were estimated at 412 nm and stated as ng/mg protein.

Results Analysis

The data were explored through one way ANOVA followed by Dunnett's multiple comparison tests with Graph pad prism 5.0 and p value < 0.05 must be deliberated as significant.

RESULTS

Evaluation of Phytochemicals

The outcomes showed methanolic extract entail of the bioactive composites like glycosides, phenols, alkaloids, flavonoids and tannins.

Toxicity Study

MM bark extract up to 2000 mg/kg ensures no death of

animals due to that 100 and 200 mg/kg body weight were selected for future studies

Nootropic study

Spontaneous alterations (% SA) in Rectangular maze

All the results were compared with disease control [**Figure 1**].The spontaneous alteration existed greatly (P<0.001) diminished in diabetic controls compared with control. The treatment group-X (200 mg/kg), standard drug treatment group IV (5 mg/kg) displays more significant effect on % spontaneous alterations (P<0.001) but group-VI (200 mg/kg) and IX (100 mg/kg) shows less significant effect (P<0.01).

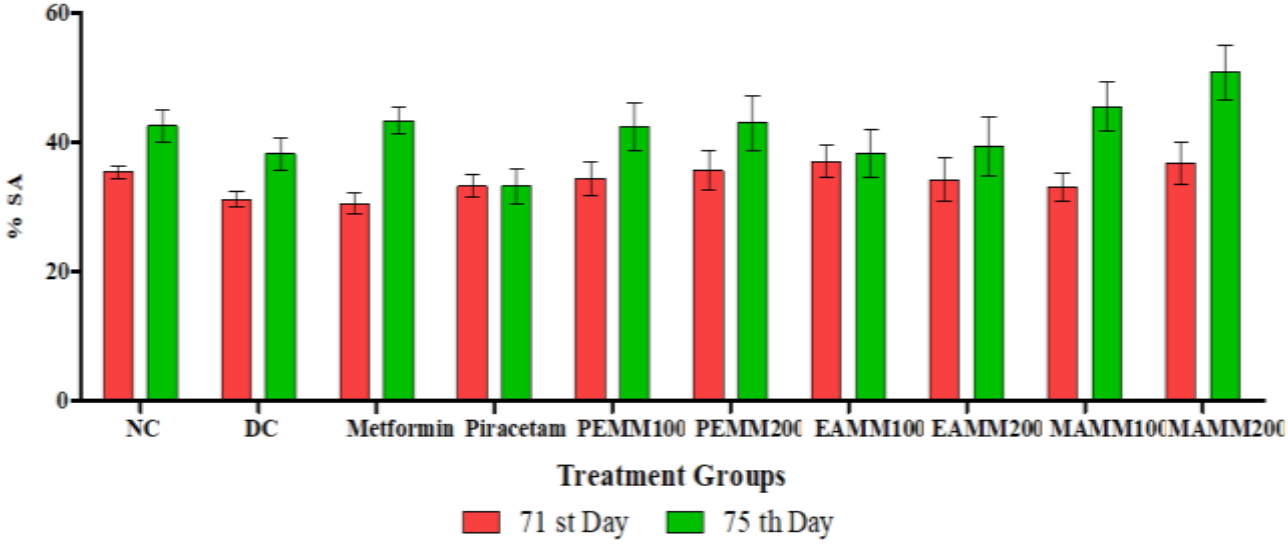

Figure 1: The effect of % SA of MM extracts

Effects of MM extracts on transfer latency (TL) in Morris water maze

The transfer latency were significantly (P<0.001) increased in diseased controls compared with control. The treatment group-X (200 mg/kg), standard drug treatment group IV (5 mg/kg) displays more significant effect in reduced transfer latency (P<0.001) but group-VI (200 mg/kg) and IX (100 mg/kg) shows less significant effect (P<0.01). All the results were compared with disease control [**Figure 2**].

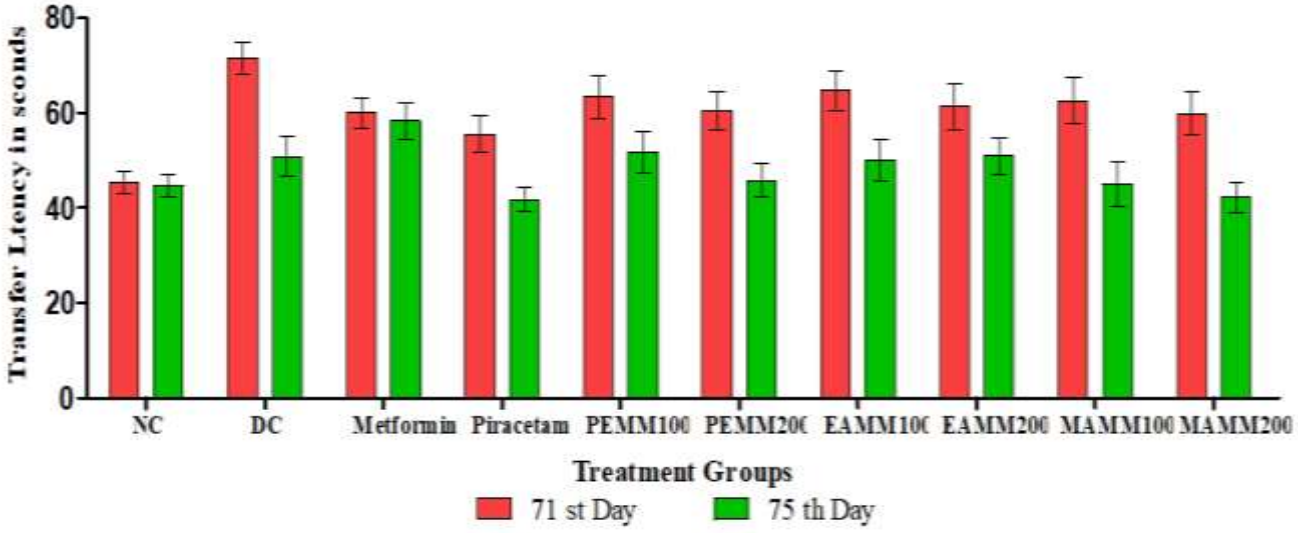

Figure 2: The effect of TL of MM extracts

Acetylcholinesterase (AChE) estimation

The AchE effectiveness were significantly increased (P<0.001) in diseased controls when compared with normal controls it suggesting cholinergic dysfunction. The treatment group-X (200 mg/kg), standard drug treatment group IV (5 mg/kg) exhibit more significantly decrease in activity (P<0.001) but group-VI (200 mg/kg) and IX (100 mg/kg) shows less significantly reduce the enzymatic activity (P<0.01). The outcomes existed were compared with diseased controls [**Figure 3**].

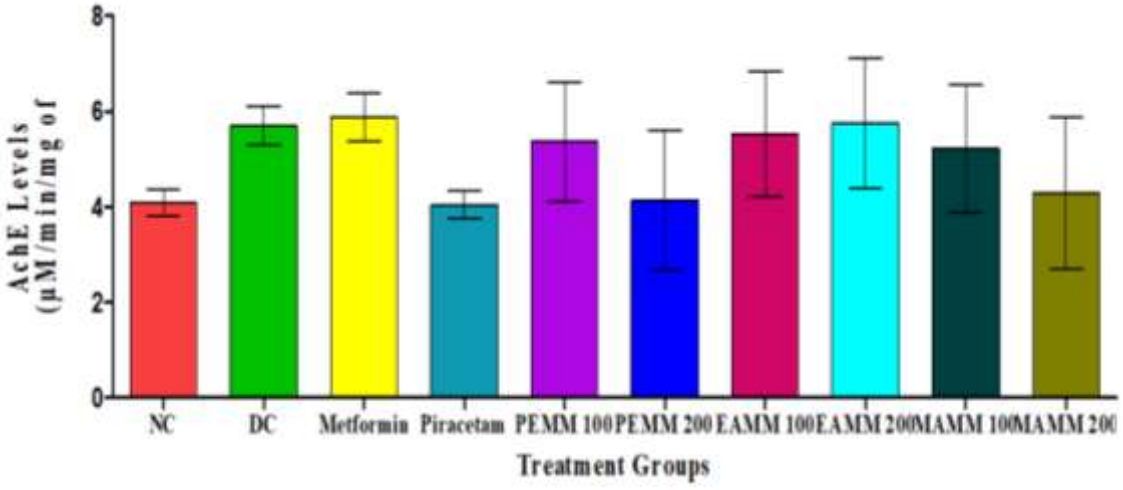

Figure 3: Effects of MM extracts on AchE activity

TBARS Assay

TBARS levels are significantly (P<0.001) augmented in diseased rats while compared to normal controls [Table **4]**. The treatment group-IV **(**5 mg/kg) and X (200 mg/kg), shows significantly (P<0.001) reduce the MDA levels. In group-VI (200 mg/kg) and IX (100 mg/kg) animals shows significantly (P<0.01) decreases MDA levels.

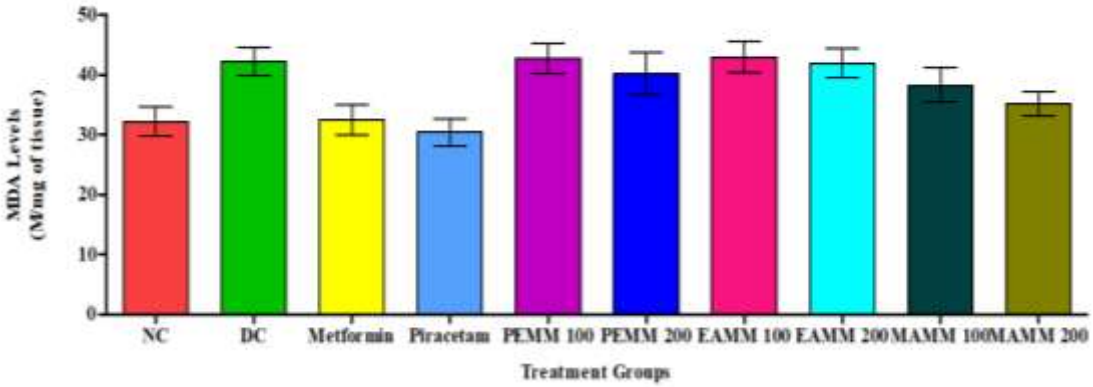

Figure 4: Effect of MM extracts on MDA levels

Nitric oxide levels

Nitric oxide levels are significantly (P<0.001) augmented in diseased rats while compared to normal controls [**Figure 5]**. The treatment group III (10 mg/kg), IV **(**5 mg/kg), and X (200 mg/kg), shows significantly (P<0.001) reduce the nitric oxide levels. In group-VI (200 mg/kg) and IX (100 mg/kg) animals shows significantly (P<0.01) decreases nitric oxide levels.

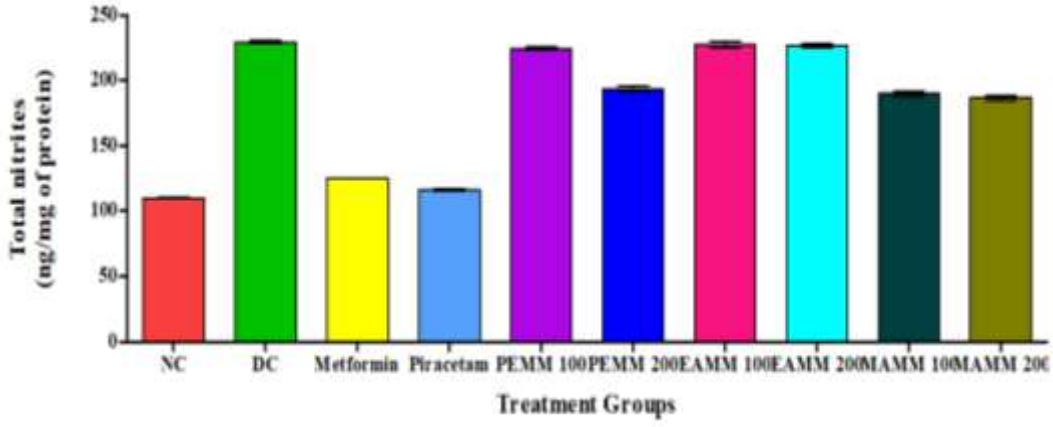

Figure 5: Effect of MM extracts on Total nitrites

CAT, SOD and GSH levels

In group-II the SOD, CAT and GSH levels were showed significantly (P<0.001) deducted when compared to control. The treatment group III (10 mg/kg), IV **(**5 mg/kg), and X (200 mg/kg), shows significantly (P<0.001) increase all antioxidants like SOD, CAT and GSH. In group-VI (200 mg/kg) and IX (100 mg/kg) animals shows significantly (P<0.01) increase [**Figure 6, 7 & 8].**

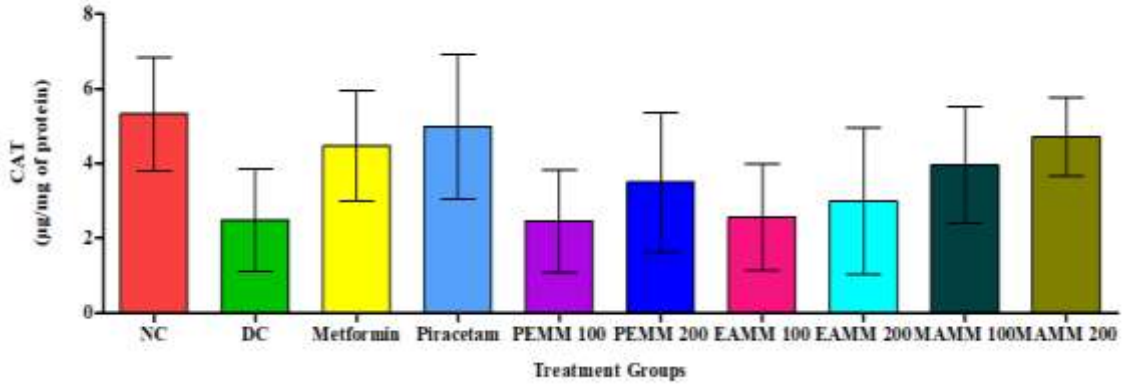

Figure 6: Effect of MM extracts on CAT levels

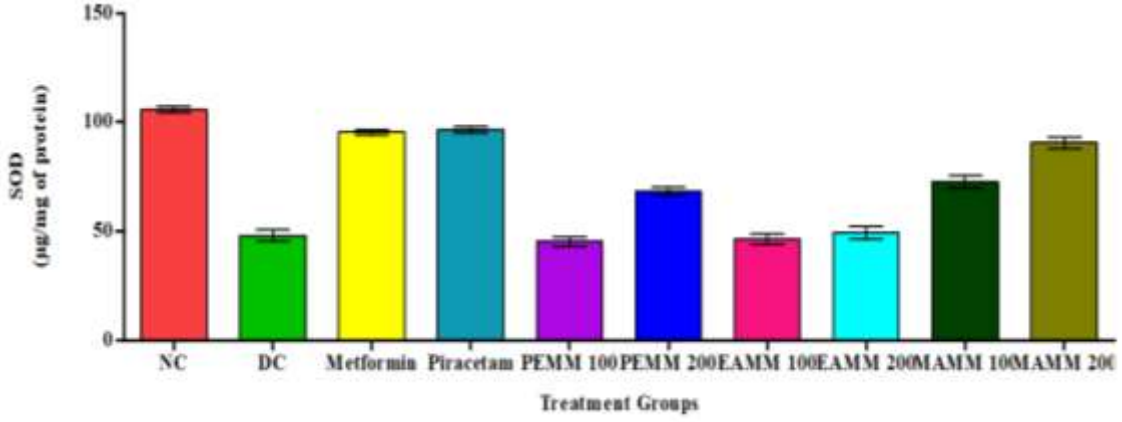

Figure 7: Effect of MM extracts on SOD levels

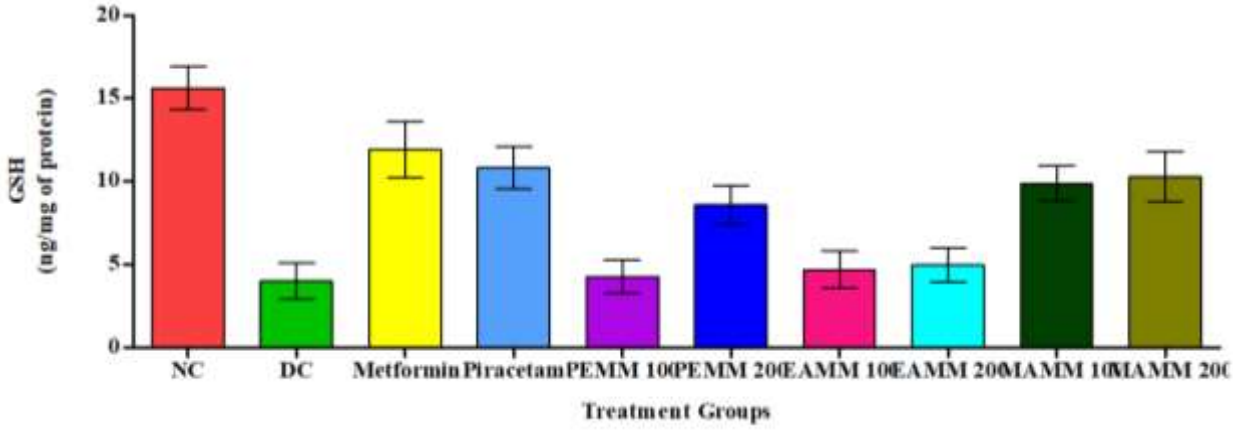

Figure 8: Effect of MM extracts on GSH levels

DISCUSSION:

Enormous quantities of plants are utilized in treatment of memory impairment. From the previous studies methanolic extract of *Myristica malabarica* leaf is evaluated for *in vitro* insulin emission studies on islets of Langerhans at concentration of 1mg/ml through inhibition of intestinal alpha-glucosidase and preserve blood glucose level through insulin secretagogues action [23]. The ethanolic extract of *Myristica malabarica* leaf extract studied anticonvulsant, antidepressant, sedative, and hypnotics [24].

The current investigation analyzed the impacts of S. grandiflora solvent extracts treatment on memory loss, oxidative stress, and cholinergic transmission impairment in chemically induced (i.e., STZ) animal model of diabetes in mice. Previous investigations have proposed that DM is associated with various neurological impairments in the focal sensory network like cognition and learning capabilities. STZ can induce type 1 or type 2 diabetes depending on the concentration used. In this present investigation, the intension is to get not exclusively the diabetes type of model, and to those additional defects in memory was also considered. Chemically STZ is a glucosamine-nitrosourea derivative, have got anti-microbial properties and found to be poisonous to the pancreatic β-cells and is used to produce exploratory diabetic condition in experimental animals. When STZ administered through the intraperitoneal routes, it creates cognition impairment and enhances cerebral masses of Amyloid-β and tau protein. STZ injection can produce the AD like pathophysiological condition in animal brain by causing the neuroinflammation and oxidative stress, which is the suitable experimental model. Moreover, the treatment of STZ causes the brain cells to become insulin-resistant, which produces the normal dementia like condition with loss of memory, progressive cholinergic deficiencies, carbohydrate hypometabolism, stress due to reactive oxygen species (ROS), and finally neurodegeneration. Subsequently, from the previously mentioned studies, it is known that STZ creates most pervasive sort of memory disability. In the current examination, STZ treated mice indicated a continual cognition decline in passive avoidance test; observed substantial reduction in step-down latency time; and in Morris water maze test, it was evidenced in increase of escape latency. The intellectual and memory decrements in DM can be resulted from hyperglycemia. Although these are multifactorial disorders, adequate information is accessible for overproduction of ROS.

Therefore, in the current study we explored the effects

of *Myristica malabarica* on diabetes (STZ) induced cognitive decline in experimental animals along with its role in oxidative stress and acetylcholinesterase activity. The chief phytoconstituents identified from the petroleum ether and ethanolic extract are alkaloids, glycoside, tannin and phenolic compound, flavonoids, protein and amino acid, phytosterols, terpenoids and carbohydrates [25]. Many phytochemicals like glycosides, phenols, alkaloids, flavonoids, and tannins were reported in the present study. The treatment group-X (200 mg/kg) methanolic extract of *Myristica malabarica* shows more significant effect on percentage spontaneous alterations ($P<0.001$) spontaneous alteration in Y maze and also exhibit more significant effect in reduced transfer latency ($P<0.001$) in morris water maze which indicates up gradation of learning and memory in STZ induced cognitive impairments. Similar works carried out in *Carica papaya* seed [26] extracts, **Clitorea ternatea** leaves [27] and *Olea europaea* [28] fruit extracts revealed significant ($P<0.001$) effect in increase in the spontaneous alterations and transfer latency in diabetes induced cognitive impairment.

Influx of acetylcholine in the hippocampus is absolutely for prepare to function memory task [29] and with a great execution on a hippocampus-reliant, unrestrained modification task [30]. In diabetes the acetylcholinesterase levels are viewed as high this catalyst hydrolyses acetylcholine present in the mind and results in intellectual decline [31]. The present study noticed a huge rise in acetylcholinesterase movement in the cerebrum of diabetic rodents. Several studies have established relationship between increase AChE activity in the brain and cognitive impairment and significantly inhibited by cinnamic acid, *Peristrophe bicalyculata*, *Clitorea ternatea* Linn and *Carica papaya* in diabetes animals [31-33].

Treatment with methanolic extract of MM significantly ($P<0.001$) increase in acetylcholinesterase activity in the brain of diabetic animals.

Under physiologic conditions, enzymatic antioxidants such as glutathione peroxide (GPX), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) as well as non-enzymatic antioxidants such as reduced glutathione (GSH), prooxidants in the body [34].

These systems are however, overwhelmed during oxidative stress conditions leading to their gradual depletion. Treatment with MM caused substantial increases in catalase and reduced gluthathione levels in treated animals compared to the untreated diabetic group. This reduction in oxidative stress markers particularly in the brain could be a factor responsible for the reversal of the DM-associated cognitive dysfunction in treated rats.

The overproduction of nitric oxide is equally lethal to neurons and nitrite level is measured as its indicator [35].

We assayed brain nitrite level in the experimental animals to establish possibility of nitrative stress as a provider to cognitive impairment in DM. Animals in the diabetic control group shows significant increase in brain nitrite level compared to control implying nitrative stress in this group. This effect was reversed with MM treatment as treated animals showed dose dependent decrease in brain nitrite levels compared to the untreated diabetic groups.

CONCLUSIONS:

Overall, natural components and extracts display antioxidant actions at central level, as well as a applicable capability to lessen vascular injury, causative overall to border neurodegeneration and cognitive resulting modifications. So, although the final fundamental mechanisms keep on mostly unidentified, they might pay to enlarge beneficial choices to treat or diminish central difficulties allied with DM.

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التحقيق في النشاط العدواني والحيوي العصبي لاستخراج لحاء *Myristica malabarica* على ضعف الإدراك الناجم عن STZ في الحيوانات التجريبية

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ملخص

تهدف الدراسة الحالية إلى تقييم مستخلصات (MM) Myristica malabarica Bark في نموذج الفئران المعرفي الناجم عن مرض السكري لنشاطه العدواني والحماية العصبية. تم تقييم نشاط تعزيز الذاكرة بواسطة اختبار المتاهة Y واختبار متاهة الماء موريس، على التوالي. تم تقييم التأثيرات العصبية الواقية لمستخلصات لحاء MM عن طريق قياس أستيل كولين استريز، بيروكسيد الدهون، ديسموتاز سوبروكسيد (SOD)، إجمالي أكسيد النيتريك ,(NO) مستويات الكاتالاز (CAT) والغلوتاثيون (GSH) في دماغ الفئران المصابة بالسكري. لوحظ أن مستخلص كحول الميثيل لحاء Myristica malabarica (MMMA) (100 و 200 مجم / كجم) يؤثر على تحسن كبير في التغيير التلقائي ($P < 0.01, P < 0.001$) ووقت الاستجابة للنقل ($P < 0.001$ $P < 0.01$) في تجارب الاحتفاظ على متاهة Y واختبار متاهة الماء Morris على التوالي. ومع ذلك، انخفاض كبير في نشاط أستيل كولين استريز($P < 0.001$)، بيروكسيد الدهون (($P < 0.001$، إجمالي ($P < 0.001$) NO وزيادة كبيرة في ,SODلوحظت مستويات CAT و ($P < 0.001$) GSH في الحيوانات المعالجة بـ 200) MMMA مجم / كجم) المتعلقة بمجموعة مكافحة مرضى السكري. تشير النتائج الحالية إلى أن مقتطفات Myristica malabarica كانت تدافع عن الانخفاض المعرفي في حالة مرض السكري والذي يتطلب بعض الدراسات الإضافية لتوضيح طريقة عملها.

الكلمات الدالة: *Myristica malabarica*، Nootropic، الحماية العصبية، تراجع الإدراك.

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Chemical Composition and Biological Evaluation of Algerian Propolis from Six Different Regions

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ABSTRACT

Propolis is considered a natural resin produced by the bee and is still used in folk medicine. Six propolis samples from *Apis mellifera* (P1-P6) collected from different regions in Algeria were investigated for their contents and biological activities. The obtained results revealed that propolis P1 exhibited the highest total phenolics (210.93 mg GAE/g propolis), total flavonoids (34.33 mg QE/g propolis), and tannins (23.36 mg CE/g propolis). For antioxidant activities, P1 showed strong free radical scavenging activity with EC_{50} values of 0.055, 0.0306, 0.109 and 0.071 mg/mL, respectively for DPPH, ABTS, **FRAP**, and phosphomolybdenum assays. On the other hand, all propolis demonstrated antibacterial activities against G+ve bacteria (*S. aureus*) with slightly higher activities that were associated with P1 and P5 (9.83 and 10.92mm, respectively). P5 exhibited the lowest MIC and MBC against *S. aureus* with values of 62.5 and 125 µg/ml, respectively. Furthermore, all propolis had moderate to low antimicrobial activities against *C. albicans* (yeast) with moderate activities for P1 and P6 (13.33 and 8.50 mm, respectively). Chemical profiling of the most bioactive propolis samples (P1, P4, and P5) using HPLC−fingerprint analysis mainly led to detecting phenolic acids and flavonoids in variable percentages.

Keywords: Propolis, antioxidants, antimicrobial, polyphenols, Algeria.

INTRODUCTION

Herbal medications are always adopted in therapeutic applications for their availability, simplicity, effectiveness, and fewer side effects relative to synthetic drugs. Propolis, also known as bee glue, is a natural substance with resinous properties and variable colors that is mainly produced by *Apis mellifera* via collecting from the exudates of multiple plant parts and their own salivary secretions [1–3].

It is basically produced for construction and the protection of bee's hive. In this sense, the Greeks came up with the propolis name that means the defense of the hive [4,5]. Historically humans applied propolis as an adhesive and embalming substance, in perfumery, and mostly in medicine and therapeutic fields [1,5] because of its antibacterial, antitumor, immunomodulatory, anti-inflammatory, antioxidant, antifungal, hepatoprotective, antidiabetic, anticancer, antiprotozoal, and antiviral activities [3,4,6–9].

About 300 compounds have been identified in propolis [3] including the phenolic compounds, which represent a wide class of organic compounds such as flavonoids, tannins and phenolic acids. Interestingly, the biological activity of propolis has been attributed to its phenolic

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ingredients [10]. Propolis has been reported to have potent antiradical and antimicrobial activities; in fact it is probably the strongest among the different bee products [11]. Propolis has been studied widely in different geographical locations since there are plenty of factors that affect its composition, such as the climate, the botanical floral and also the extraction process [12,13].

Overproduction and accumulation of reactive species within the human body lead to a phenomenon recognized as oxidative stress that initiates several health disorders like cancer, cardiovascular diseases, and inflammation. The destructive effects of such species can be diminished via utilizing naturally occurring antioxidant agents as free radical scavengers [14–17].

Additionally, the emergence of resistant pathogenic strains that fail to respond to existing drugs poses a huge challenge for health care providers and current research has been redirected to discover new antibiotics. Natural sources like medicinal plants, microbial extracts, and marine organisms [18,19] were extensively studied for the discovery of new safe and effective antibiotics to counteract the resistance problem. Moreover, several naturally occurring bioactive compounds have been reported for their antimicrobial effects against different microbial infections [20,21]. Therefore, this study aims to investigate the Algerian raw propolis samples collected from different areas for their chemical profiles as well as their antioxidant and *in vitro* antimicrobial activities.

MATERIALS AND METHODS

Propolis samples

Six samples of raw propolis were harvested from the wild from six different regions in Algeria namely: Tipaza (P1; Latitude: 36.59°N; Longitude: 2.44°E), Blida (P2; Latitude: 36.47°N; Longitude: 2.83°E), Bouira (P3; Latitude: 36.37°N; Longitude: 3.90°E) which locate in the north, Batna (P4; Latitude: 35.56°N; Longitude: 6.19°E) in the east, **Sidi-Bel-Abbes** (P5; Latitude: 35.21°N; Longitude: 0.63°W) in the west, and **Ghardaïa (P6;** Latitude**:** 32.49°N; Longitude**:** 3.64°E**) in the** Northern desert. Samples were collected during spring and winter of 2019. The samples were kept at 4°C until extraction, biological and chemical investigations were performed.

Chemicals and reagents

All solvents, standards and reagents were of highly analytical grade. Ethanol, Folin-Ciocalteu's reagent, Na_2CO_3, gallic acid, $AlCl_3$, quercetin, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) **and ascorbic acid** were obtained from Sigma-Aldrich (Steinheim, Germany). $ABTS^+$(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulphate, BHT, Trolox, **$K_3[Fe(CN)_6]$,** trichloroacetic acid, $FeCl_3$, sulfuric acid, sodium phosphate and ammonium molybdate were obtained from Fluka Chemicals. Nutrient agar and Nutrient Broth media were purchased from HiMedia Laboratories Pvt. Ltd (Mumbai, India).

Extract preparation

The propolis was grated first, and then each sample of 1 g was dissolved in 30 mL of ethanol (70%) in a 50 mL flask and left for 96 hours at room temperature. Afterward, the mixture was filtered and the extraction was repeated. The two extracts were combined and diluted to 100 mL with 70% ethanol in a volumetric flask. Next the hydro-alcoholic extracts were analyzed to determine the total phenolics and flavonoids [22].

Total phenolic contents

Total phenolic contents in each tested extract were determined by the Folin-Ciocalteu's [23] method with minor modifications. Hydro-alcoholic extracts (0.1 mL) were mixed with 0.5 mL of Folin-Ciocalteu's reagent (10%) and 0.4 mL of (7.5%) Na_2CO_3, and the absorbance was measured at 765 nm after 30 min of incubation at room temperature. The total polyphenol content was calculated based on a standard curve prepared using gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of sample.

Total flavonoids contents

Total flavonoid contents in each tested extract were

determined according to the reported procedures [24] with minor modification. An amount of 0.5 mL of $AlCl_3$ (2%) was added to 0.5 mL of extract, after 1 h the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin equivalent (mg QE/g) using a calibration curve.

Total tannins contents

Total tannins content were determined as previously described by [25]. Briefly, 50µL of the extract was added to 1500µL of vanillin-methanol solution (4%) and 750µL of concentrated hydrochloric acid, 20 min later the mixture was measured at 510 nm. The catechin solution was used as standard and treated the same manner.

2,2-diphenyl-1-picrylhydrazyl radical (DPPH) free radical

Various concentrations of each sample (100 µL) were added to DPPH-ethanol solution (3900 µL, 60 µM) as previously described [26] with minor alterations. After an hour of incubation, the absorbance was measured at 517 nm. **Ascorbic acid was selected** as an antioxidant reference and treated in the same manner, and the calculation was carried out via finding the inhibition percentage (I%), $I\% = [(A_0 - A_i/A_0)*100]$; A_0: Absorbance of DPPH free radical, A_i: Absorbance of the free radical with the antioxidant, and the EC_{50} (Half maximal effective concentration was estimated.

$ABTS^+$ free radical-scavenging activity

$ABTS^+$(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging evaluation was based on a previously published report [27]. Accompanying with ascorbic acid, BHT, and Trolox were used as antioxidant references. Initially the $ABTS^+$ radical with the absorbance Abs_{734nm}: 0.7 was prepared by reacting ABTS-aqueous solution (7mM) with the persulfate-ethanol solution (2.45 mM) during 16 hours in the dark, then 50 µL at different concentrations of the samples was added to 950 µL $ABTS^+$, and measured at 734 nm. Both I% and EC_{50} were adopted for the calculations.

Ferric reducing antioxidant assay

According to previous reports [28], 50µL of each sample with various concentrations were added to 500 µL of **Phosphate buffer solution (200mM, pH=6) and 500 µL of $K_3[Fe(CN)_6]$(1%) with 30s of shaking and incubation at 50°C in a water bath for 20 min,** Trichloroacetic acid (**500 µL,** 10%) was added to the previous mixture, **then** 500 µL of the supernatant of the last solution was mixed with water (500 µL) and $FeCl_3$ (100 µL, 0.1%). The absorbance was measured at 700 nm against a blank consisting of the same reagents with only ethanol 70% instead of samples, using ascorbic acid, BHT and Trolox as antioxidant references and the same calculation parameters.

Phosphomolybdenum total antioxidant capacity

The phosphomolybdenum scavenging activity was based on phosphomolybdenum reagent and each of **ascorbic acid,** BHT, and Trolox as antioxidant references. 0.1 mL of each sample was mixed with 1 mL of Phosphomolybdenum reagent [100 mL of sulfuric acid (0.5 mM), 100ml of sodium phosphate (28 mM) and 100 mL of ammonium molybdate (4mM)]. The reaction was carried out in the dark for 90 min under 95°C in a water bath, the absorbance was measured at 695 nm [29]. The same parameters of EC_{50} were used for the calculation.

Antimicrobial activity

The antimicrobial activity of the samples was investigated by the agar disc diffusion method. Four different test microbes namely: *Staphylococcus aureus* (G+ve bacteria), *Escherichia coli* (G-ve bacteria), *Candida albicans* (yeast), and *Aspergillus niger* (fungus) were used. Nutrient agar plates were heavily seeded uniformly with 0.1 mL of 10^5-10^6 cells/mL in case of bacteria and yeast. A Czapek-Dox agar plate seeded by 0.1 mL the fungal inoculum was used to evaluate the antifungal activities. The plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. The plates were then incubated at 37°C for 24 hours for bacteria and at 30ºC for 48 hours. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter (mm). The experiment was carried out more than once and mean of readings was recorded [18].

MICs and MBCs evaluation

Staphylococcus aureus ATCC 6538 (G+ve bacteria) and *Escherichia coli* ATCC 25922 (G-ve bacteria) were used to evaluate the MIC values of the potent active fractions/compounds. The test strains were cultivated in 100 ml bottle with each test at 35°C for 24 hours on Mueller Hinton medium. Bacterial cells were collected by centrifugation at 5000rpm under aseptic conditions at 4°C and the cells were washed using sterile saline till the supernatant becomes clear. Cell suspension has been performed to achieve optical density of 0.5 to 1 (at 550 nm) giving actual colony forming units of $5x10^6$ cfu/ml. Resazurin solution was prepared by dissolving 270 mg tablet in 40 ml of sterile distilled water. 96-well sterile-microplates were prepared. 50 µl of test material in methanol was pipetted into the first row of the plate. 10 µl of Resazurin indicator solution was added followed by 10 µl of bacterial suspension. The plates were prepared in duplicate and placed in an incubator set at 37°C for 18–24 hours. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value. MBC has been done by streaking of the two concentrations higher than MIC and the plates exhibiting no growth were considered as MBC [30].

HPLC conditions

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid was added to acetonitrile (B) which does not affect the separation column at a flow rate 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (82% A) ; 15-16 min (82% A) and 16-20 (82%A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 µl for each of the sample solutions. The column temperature was maintained at 40 °C [31,32].

RESULTS

Total polyphenolic, flavonoid and tannins content in propolis extracts

According to Table 1, the phenolic contents in Algerian propolis ranged from 45.37 ± 11.01 to 210.93 ± 36.02 (mg GAE/g propolis) in P6 to P1 samples orderly, and the total flavonoid contents varied from 07.32 ± 0.11 to 34.33 ± 0.44 (mg QE/g propolis) relating to P4 and P1. In general, the propolis in northern areas of Algeria P1, P2, and P3 have higher content of both phenolics and flavonoids, especially the sample from Tipaza (P1). For the tannins, the content varied between 3.77 to 23.36 (mg CE/g propolis) in samples P6 and P1.

The antiradical activities of propolis extracts

Concerning the antioxidant activities, the EC_{50} parameter was used for all antioxidant activities assays. Table 1 shows that the EC_{50} of antiradical activities oscillated between 0.055-0.59 mg/mL (DPPH), 0.0033-0.354 mg/mL (ABTS), 0.109-0.377 mg/mL (FRAP), and from 0.055 to 0.47 mg/mL (phosphomolybdenum), these results indicate that samples P1 and P3 are the strongest antioxidants relative to the other samples. Sample P3 from Bouira region had a good capacity against the ABTS free radical which was estimated with 0.0033 mg/mL and it seems to be a very powerful antioxidant. As shown in Table 1 the value 0.109 mg/mL in both P1 and P3 had the highest values. For the phosphomolybdenum activity in table 1 all the five samples presented an intense capacity except the sample P6 in south region.

Table 1: Total polyphenolic, flavonoid and tannins contents, and antiradical activities of Algerian propolis extracts

Test/ Bio-assay	Tested propolis samples/ Standards								
	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**Ascorbic acid**	**Trolox**	**BHT**
Total phenolic (mg GAE/g propolis)[1,2]	210.93 ± 36.02	107.56 ± 22.78	183.15 ± 15.18	56.65 ± 10.32	57.04 ± 9.37	45.37 ± 11.01	-	-	-
Total flavonoid (mg QE/g propolis)[3]	34.33 ± 0.44	29.16 ± 0.27	18.64 ± 0.63	07.32 ± 0.11	19.04 ± 0.31	09.52 ± 0.13	-	-	-
Total tannins (mg CE/g propolis)[4]	23.36 ± 1.91	6.53 ± 0.58	13.74 ± 0.82	23.17 ± 3.97	6.72 ± 0.91	3.77 ± 1.24	-	-	-
EC_{50} (DPPH)[5] mg/mL	0.055 ± 0.001	0.205 ± 0.007	0.065 ± 0.003	0.59 ± 0.001	0.27 ± 0.002	0.34 ± 0.011	0.124 ±0.001	0.0042 ±0.0001	0.0025 ±0.0002
EC_{50} (ABTS) mg/mL	0.0306 ± 0.0014	0.088 ± 0.0041	0.0033 ± 0.001	0.354 ± 0.007	0.106 ± 0.0081	0.158 ± 0.010	0.004 ± 0.0001	0.0058 ±0.0001	0.0043 ±0.0005
EC_{50} **(FRAP)** mg/mL	0.109 ± 0.01	0.178 ± 0.026	0.109 ± 0.012	0.311 ± 0.072	0.294 ± 0.009	0.377 ± 0.062	0.0072 ± 0.001	0.0056 ± 0.001	0.013 ± 0.003
EC_{50} (Phosphomolybdenum) mg/mL	0.071 ± 0.0014	0.078 ± 0.0035	0.055 ± 0.0077	0.064 ± 0.0028	0.125 ± 0.007	0.47 ± 0.014	0.023 ±0.0014	0.027 ±0.0021	0.155 ±0.0012

[1]Results are (means ± S.D.) (n = 3)
[2]GAE: Gallic acid equivalent
[3]QE: Quercetin equivalent
[4]CE: Catechin equivalent
[5]EC_{50}: Half maximal effective concentration

The antimicrobial activity of propolis extracts

The antimicrobial activity of the extracts was assessed against *Staphylococcus aureus* (G+ve bacteria), *Escherichia coli* (G-ve bacteria), *Candida albicans* (yeast), and *Aspergillus niger* (fungus) through the measurement the diameter of inhibition zone, the results in Table2 indicated that the hydro-alcoholic extracts of propolis are positively effective against the *Staphylococcus aureus*, *Candida albicans*, and non-effective considering *Escherichia coli* and *Aspergillus niger* except for the sample P4 which is effective against the fungus.

Table 2: The antimicrobial activity of propolis extracts compared to standard antibiotics

Samples	Clear zone (ϕmm)			
	Staphylococcus aureus	***Escherichia coli***	***Candida albicans***	***Aspergillus niger***
P1	9.83 ± 0.76	0	13.33 ± 0.58	0
P2	8.17 ± 0.29	0	8.16 ± 0.29	0
P3	9.33 ± 0.57	0	8.33 ± 0.58	0
P4	7.50 ± 0.50	0	6.97 ± 0.06	7.33 ± 0.58
P5	10.92 ± 0.14	0	8.0 ± 0.0	0
P6	8.67 ± 0.58	0	8.50 ± 0.50	0
Neomycin50ug/ml	23.50 ± 0.50	19.83 ± .76	19.17 ± 0.29	0
Cyclohexamide 50ug/ml	0	0	0	22.17 ± 0.76

P: Propolis. mm: Millimeter.

MIC and MBC determination

Results in Table 3 explained that extract P5 exhibited the lowest MIC and MBC against *S. aureus* with values of 62.5 and 125 µg/ml, respectively followed by extracts P1 (125&250 µg/ml) and P3 (250 &325 µg/ml). For *E. coli* the MIC and MBC value for all extracts were high but extract P5 had moderate values of MIC and MBC (250 and 500 µg/ml, respectively).

Table 3: The minimum inhibitory concentrations (MICs), and minimum bactericidal concentrations (MBCs) of the most active selected extracts

Extracts	Pathogenic microorganisms			
	***S. aureus* ATCC 6538**		***E. coli* ATCC 25922**	
	MIC (µg/ml)	**MBC (µg/ml)**	**MIC (µg/ml)**	**MBC (µg/ml)**
P1	125	250	250	750
P3	250	325	500	750
P5	62.5	125	250	500

MIC: Minimum Inhibitory Concentration.

ATCC: American Type Culture Collection.

HPLC-fingerprint analysis of propolis samples

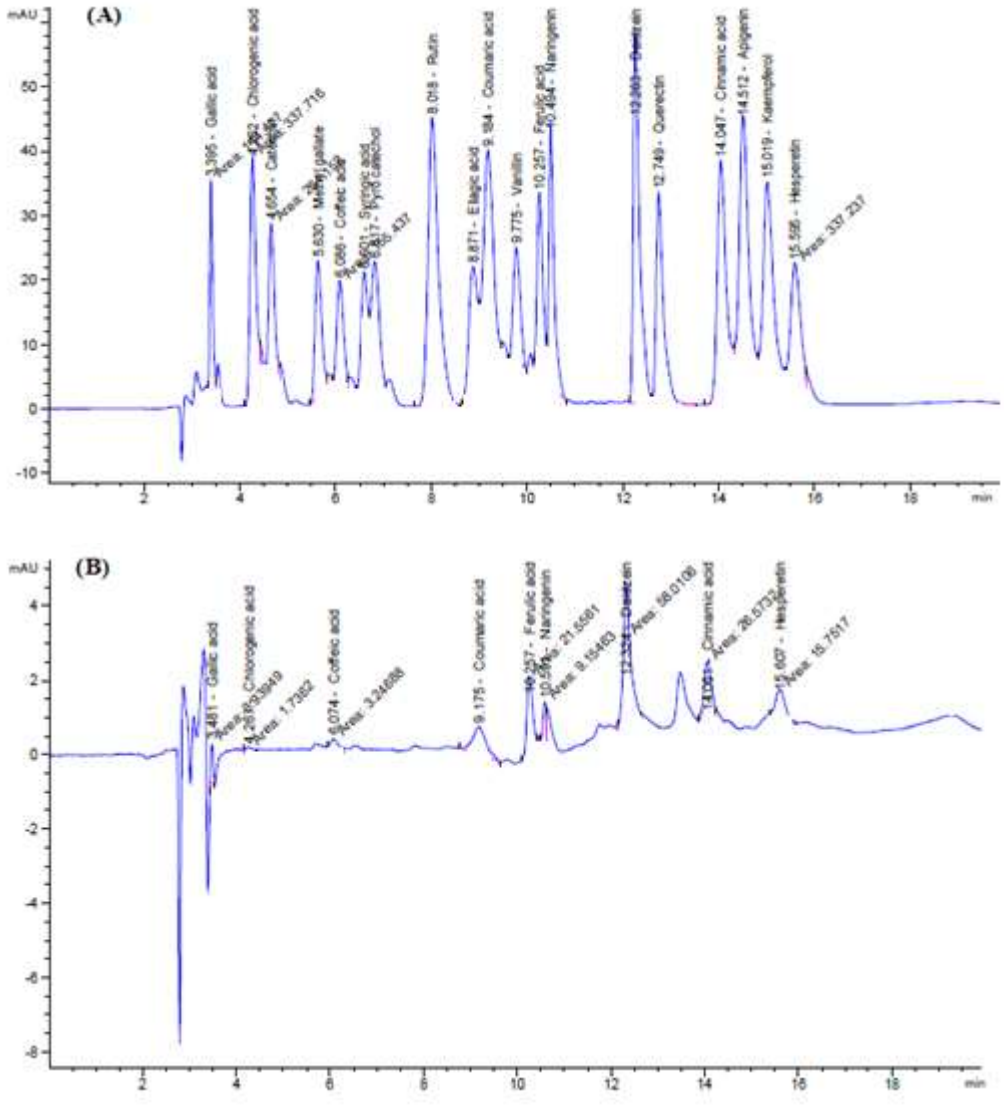
In this research work, a proper HPLC-fingerprint approach has been established to determine the chemical components in the most bioactive Algerian propolis samples (P1, P4, and P5). The obtained HPLC chromatograms of the investigated extracts were compared to nineteen standard phenolic compounds (Table 4 and Figure1).

Table 4: Areas under peaks and concentrations of the identified phenolic compounds in three propolis samples (P1, P4, and P5) compared to nineteen standard phenolic compounds

Polyphenol STD			P1				P4				P5			
Standards	**Conc. (µg/ml)**	**Area%**	**R_t (min)**	**Area %**	**Conc. (µg/ml= µg/200mg)**	**Conc. (µg/g)**	**R_t (min)**	**Area%**	**Conc. (µg/ml= µg/200mg)**	**Conc. (µg/g)**	**R_t (min)**	**Area %**	**Conc. (µg/ml= µg/200mg)**	**Conc. (µg/g)**
Gallic acid	15	2.1465	3.48	3.94	0.41	2.05	3.48	3.64	0.38	1.89	3.48	3.75	0.39	1.95
Chlorogenic acid	50	5.0193	4.26	1.74	0.26	1.29	4.25	2.35	0.35	1.74	4.28	1.60	0.24	1.19
Catechin	75	3.9259	4.65	ND	ND	ND	4.65	ND	ND	ND	4.65	ND	ND	ND
Methyl gallate	15	2.9489	5.63	ND	ND	ND	5.72	9.88	0.75	3.73	5.49	1.46	0.11	0.55
Caffeic acid	18	2.3102	6.07	3.25	0.38	1.88	6.08	8.52	0.99	4.94	5.85	3.59	0.42	2.08
Syringic acid	17.2	2.6503	6.60	ND	ND	ND	6.60	ND	ND	ND	6.60	ND	ND	ND
Pyrocatechol	40	3.9140	6.81	ND	ND	ND	6.83	2.32	0.35	1.76	6.81	ND	ND	ND
Rutin	61	10.1941	8.01	ND	ND	ND	8.01	ND	ND	ND	8.01	ND	ND	ND
Ellagic acid	120	3.6625	8.87	ND	ND	ND	8.51	2.85	1.39	6.93	8.87	ND	ND	ND
Coumaric acid	20	7.900	9.17	14.93	0.56	2.81	9.18	4.85	0.18	0.91	9.18	9.77	0.37	1.84
Vanillin	12.9	2.7036	9.77	ND	ND	ND	9.74	1.54	0.11	0.55	9.77	ND	ND	ND
Ferulic acid	20	3.6901	10.25	21.56	1.74	8.68	10.25	ND	ND	ND	10.25	ND	ND	ND
Naringenin	30	4.5775	10.59	9.15	0.89	4.46	10.59	39.84	3.88	19.41	10.59	12.75	1.24	6.21
Daidzein	35	8.1338	12.32	58.01	3.71	18.55	12.31	61.95	3.96	19.81	12.34	29.29	1.87	9.36

Polyphenol STD			P1				P4				P5			
Standards	**Conc. (µg/ml)**	**Area%**	**R_t (min)**	**Area %**	**Conc. (µg/ml= µg/200mg)**	**Conc. (µg/g)**	**R_t (min)**	**Area%**	**Conc. (µg/ml= µg/200mg)**	**Conc. (µg/g)**	**R_t (min)**	**Area %**	**Conc. (µg/ml= µg/200mg)**	**Conc. (µg/g)**
Quercetin	40	5.2241	12.74	ND	ND	ND	13.15	1.41	0.16	0.80	12.63	40.36	4.59	22.96
Cinnamic acid	10	7.5052	14.06	26.57	0.53	2.63	14.06	42.60	0.84	4.22	14.08	20.99	0.42	2.08
Apigenin	50	10.0262	14.51	ND	ND	ND	14.52	29.99	2.22	11.12	14.49	28.66	2.12	10.62
Kaempferol	60	8.4556	15.01	ND	ND	ND	15.0	11.57	1.22	6.10	15.01	ND	ND	ND
Hesperetin	20	5.0122	15.60	15.75	0.93	4.67	15.62	80.57	4.78	23.89	15.58	16.65	0.99	4.94

R_t: Retention time. ND: Not Detected.



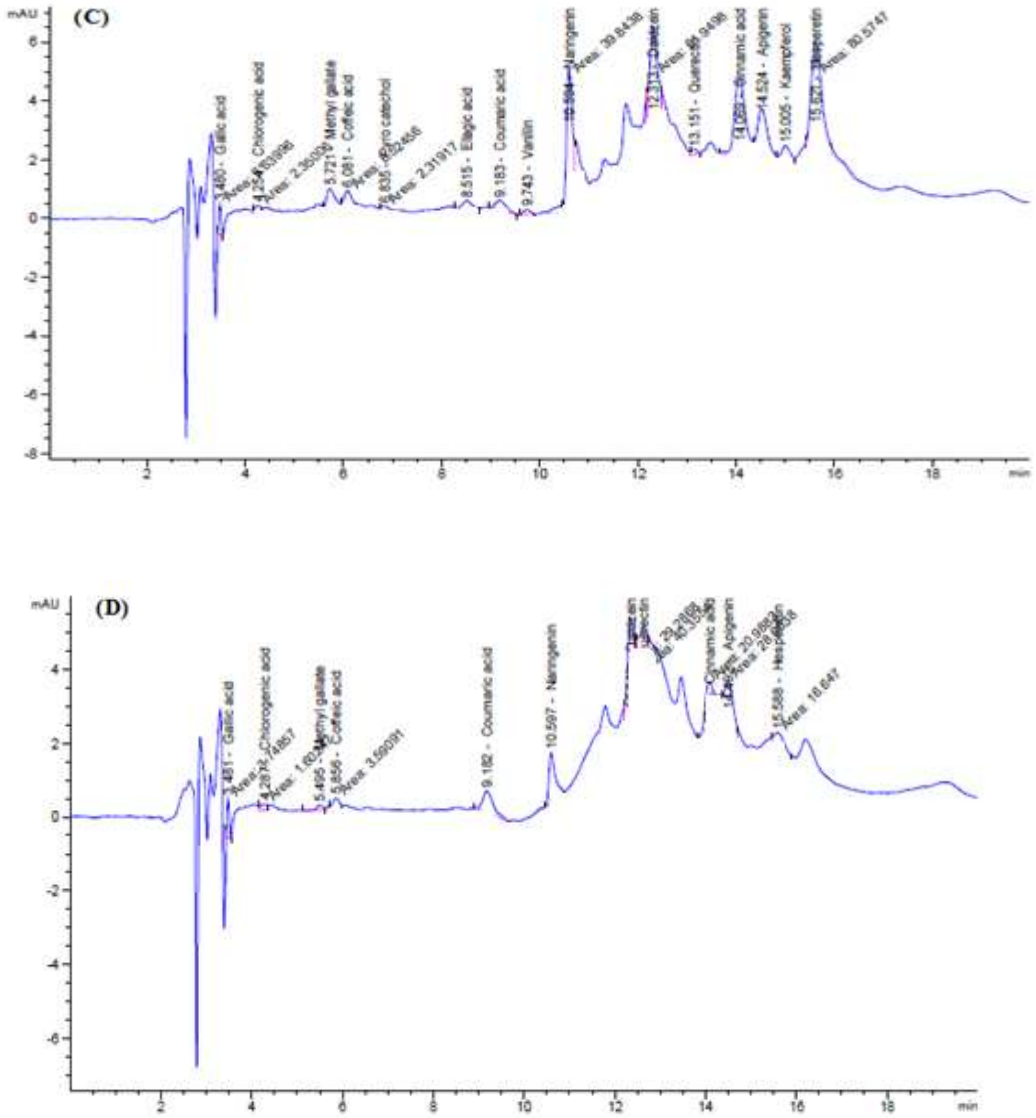


Fig.1. (A) HPLC chromatogram of standard phenolic compounds; (B) HPLC chromatogram of P1; (C) HPLC chromatogram of P4 and(D) HPLC chromatogram of P5.

DISCUSSION

Regarding the extraction procedures, the ethanol 70% solvent is commonly used for the phenolic extraction considering the solubility concept and it is more effective than water, less toxic than methanol with advantage for dewaxing purposes [33]. Based on previous studies the extraction was carried out in darkness at the room temperature to reduce possible degradation of the matter that may result from agitation.

The phenolic results of Algerian propolis (45.37- 210.93 mg GA/g propolis) are similar to the range of Morocco (77.89-241.66 mg GAE/g) [34], Kashmir Himalaya region (180-260 mg GAE/g) [35], Poland (150.05 to 197.14 mg/g GAE) [36], and Indian propolis (159.10-269.10) [37]. As for the flavonoids, the amounts (7.32-34.33 mgQE/g propolis) at most are in the same range of west Algeria and Ethiopia

reported values [25,38] but mainly are less than many other countries. The tannins content in propolis was not widely analyzed by researchers probably due to their low abundance. This large variation in phenol, flavonoid and the tannins amounts, whether between Algerian regions or comparing with other parts of the world suggests that the geographical locations including the botanical floral affect the quantification of propolis [12], in addition to the climate and the harvesting time factors [22].

The highest value in DPPH free radical-scavenging activity (P1) among these samples is close to the findings of the south of Portugal, Kashmir Himalaya and India [22,39]. The synthetic radical $ABTS^{\bullet+}$ with the blue-green color becomes pale after turning it into a stable form and gaining an electron from the antioxidant agent [40]. The FRAP test is similar to ABTS except that it done under acidic pH instead of neutral conditions, the FRAP process reduces ferric-tripyridyltriazine $[FeIII(TPTZ)]^{3+}$ to a ferrous complex $[FeII(TPTZ)]^{2+}$ with a blue color. It is known that the antioxidant activity is related to the phenolic compounds including the flavonoids [10], therefore we report the diversity of the capacity between locations and in the activity type as well, which explains why the extracts with the highest amounts in phenolic P1, P3 have more potent antioxidant properties relative to the other investigated samples.

Regarding the antimicrobial activitity of the tested propolis samples, the current findings come in good agreement with many published reports that have demonstrated the effectiveness of propolis against Gram-positive bacteria and *Candida albicans* while inactive against Gram-negative bacteria [41].

HPLC-fingerprint approach is a well-known method was utilized for the determination of phenolic profiles in many plant extracts [31,32]. In the current study, the tested propolis samples showed a variable content of phenolic compounds, this is due to several factors, including Ecological conditions. Reviewing the literature revealed that *HPLC-UV analysis of Algerian propolis led to identification of six phenolic compounds including pinostrombin chalcone (38.91%), galangin (18.95%), naringenin (14.27%), tectochrysin (25.09%), methoxychrysin (1.14%) and suberosin (1.65%) [42]. The ethanolic extract of Uruguayan propolis was investigated for its phenolic composition via using RP-HPLC. The results revealed the presence of gentistic and p-coumaric acids as well as 8 flavonoidal compounds namely fisetin, myricetin, luteolin, quercetin, kaempferol, pinocembrin, chrysin and tectochrysin [43].* RP-HPLC analysis of water extract of Brazilian propolis revealed the presence of phenolic acids like caffeic acid, *p*-coumaric acid and *trans*-cinnamic acid [44]. Eight polyphenolic compounds were detected by HPLC-UV in the 80% methanol extract of Chinese propolis *viz.* caffeic acid, isoferulic acid, 3,4-dimethoxycinnamic acid, pinobanksin 5-methyl ether, pinocembrin, benzyl caffeate, chrysin and galangin [45]. Rutin, quercetin, apigenin, kaempferol, chrysin and caffeic acid were detected in different aqueous ethanolic extracts of Romanian propolis using HPLC analysis [46,47] reported that 21 flavonoidal compounds and two caffeic acid esters were identified by HPLC in the 70% ethanol extract of Egyptian propolis and its sub-fractions including luteolin, apigenin, chrysin, acacetin, chrysin-7-methylether, luteolin-3'-methylether, myricetin, galangin, naringenin, hesperetin, genistein, dimethylallylcaffeate, and phenylethylcaffeate. Our current findings are matched with study of Shashikala and his Co-workers, which stated that HPLC-fingerprint analysis of the 70% ethanol extract of Indian propolis led to identification of p-coumaric acid, ferulic acid, epicatechin, gallic acid, caffeic acid and quercetin [48]. HPLC-UV/DAD analysis of Italian propolis hydroalcoholic extract revealed the presence of phenolic acids and their derivatives including caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethyl-caffeic acid, cinnamic acid, caffeic acid prenyl ester, caffeic acid benzyl ester, caffeic acid phenylethyl ester, *p*-coumaric prenyl ester, *p*-coumaric benzyl ester, caffeic acid cinnamyl ester, p-coumaric cinnamyl ester, and p-

methoxy cinnamic acid cinnamyl ester. Also, the results revealed the presence of flavonoides like quercetin, quercetin-3-methyl-ether, chrysin-5-methyl-ether, apigenin, kaempferol, isorhamnetin, galangin-5-methyl-ether, quercetin-7-methyl-ether, chrysin, and galangin [49]. HPLC analysis of ethanolic extract of Croatian propolis allowed the identification of caffeic acid, naringenin, chrysin, pinocembrin, and galangin [50]. HPLC-UV/DAD investigations of Chinese propolis 80% methanol extract led to characterization of rutin, quercetin, luteolin, genistein, galangin and curcumin [51]. UHPLC-DAD analysis of the Indian propolis extract allowed the quantification of caffeic acid, *trans*-ferulic acid, *p*-coumaric acid, quercetin, luteolin, naringenin, apigenin, kaempferol, pinocembrin, CAPE, pinobanksin-3-*O*-acetate, acacetin, and galangin [52-54].

In conclusion, propolis, natural resins produce by bees, is considered as a promising source for the isolation of different groups of compounds such as phenolics, flavonoids as well as tannins with clinical value for the treatment of certain medical conditions.

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التركيب الكيميائي والتقييم البيولوجي للبروبوليس الجزائري من ست مناطق مختلفة

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ملخص

يعتبر البروبوليس راتينجًا طبيعيًا ينتجه النحل ولا يزال يستخدم في الطب الشعبي. تم فحص ست عينات بروبوليس من نحل العسل الغربي (P1-P6) تم جمعها من مناطق مختلفة في الجزائر لمعرفة محتوياتها وأنشطتها البيولوجية. أظهرت النتائج المتحصل عليها أن البروبوليس P1 أظهر أعلى نسبة من الفينولات الكلية (210.93 مجم من GAE / جم بروبوليس)، وفلافونويد كلي (34.33 مجم QE / جم بروبوليس)، والتانين (23.36 مجم / جم بروبوليس). بالنسبة للأنشطة المضادة للأكسدة، أظهر P1 نشاطًا قويًا في إزالة الجذور الحرة بقيم EC_{50} تبلغ 0.055 و 0.0306 و 0.109 و 0.071 مجم / مل على التوالي لمقايسات DPPH و ABTS و FRAP و phosphomolybdenum. من ناحية أخرى، أظهرت جميع أنواع البروبوليس نشاطًا مضادًا للبكتيريا ضد بكتيريا (*S. aureus*) G + ve مع أنشطة أعلى قليلاً مرتبطة بعينات P1 و P5 (9.83 و 10.92 مم، على التوالي). أظهر P5 أدنى MIC وMBC ضد *S. aureus* بقيم 62.5 و 125 ميكروجرام / مل على التوالي. علاوة على ذلك ، كان لجميع عينات البروبوليس أنشطة متوسطة إلى منخفضة كمضادات للميكروبات ضد*C. albicans* (الخميرة) مع أنشطة متوسطة لعينات P1 و P6 (8.50 و13.33مم، على التوالي). كما أدى التنميط الكيميائي لعينات البروبوليس الأكثر نشاطًا بيولوجيًا (P1 و P4 و P5) بإستخدام تحليل بصمات الأصابع (HPLC-fingerprint) بشكل أساسي إلى إكتشاف الأحماض الفينولية والفلافونويدات بنسب متغيرة.

الكلمات الدالة: البروبوليس، مضادات الأكسدة، مضادات الميكروبات، عديد الفينولات، الجزائر.

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The Efficacy and Tolerability of the Use of Combined Versus Single Analgesic and Prophylactic Medications in Severe Migraine

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ABSTRACT

Background: Migraine is a common cause of primary headaches that may interfere with normal daily activities for which different modalities of treatment had been used. In the present study different types of analgesics and prophylactic medications had been evaluated for their efficacy and tolerability.

Objective: To evaluate the efficacy and tolerability of: 1. Combined analgesic, Excedrin (aspirin with paracetamol and caffeine) in comparison with single analgesic, diclofenac, as a therapy for severe migraine attack.

2. Combined prophylactic, amitriptyline, or propranolol with pizotifen in comparison with pizotifen alone as prophylactic drugs for severe migraine.

Materials & Methods: Part (1): 80 patients with severe migraine were enrolled and randomly assigned to receive an oral tablet of either Excedrin (aspirin 250 mg, acetaminophen 250mg, caffeine 65mg) four times daily when needed (n=40) or diclofenac 50mg twice daily when needed as abortive treatment for migraine attack, in addition to the use of pizotifen twice a day in both subgroups for 10 days duration. Part (2): 46 patients who showed good response from part one of the study were divided randomly into three subgroups with different prophylactic regimens.

1. oral pizotifen 0.5mg tablet twice a day, n=16.
2. oral pizotifen 0.5mg twice a day with propranolol 20mg tablet twice a day, n=15.
3. oral pizotifen 0.5mg tablet twice a day with amitriptyline 10mg tablet once at night. n=15.

Efficacy was assessed by determining the patients' number exhibiting improvement with no or mild headache. Tolerability is no or minimal side effects or interactions.

Results: Part (1): A good response to treatment was obtained in 30% of the diclofenac group vs 85% of the Excedrin group (P<0.01). Part (2): A good response to treatment was obtained in 33.3% of the pizotifen group vs 60% of pizotifen with propranolol group (P< 0.05) vs 87.5% of pizotifen with amitriptyline group (P< 0.01).

Conclusion: Combined medications are more effective than single medications in the treatment & prophylaxis of severe migraine.

Keywords: Excedrin, Diclofenac, Amitriptyline, Propranolol, Pizotifen, Severe migraine.

INTRODUCTION:

Migraine is more than a headache. It is a neurological disease with considerable social and economic impact. It affects approximately 15% of women and 6% of men. It is defined as a benign and recurring syndrome of headache, nausea, vomiting, and/or other symptoms [1,2,3,4,5,6,7,8,9].

It usually begins in childhood, adolescence, or adult life and recurs with diminishing frequency during advancing years. [2,4,6,10,11,12,13]

Migraine can often be recognized by its active activators (red wine, stress, menses, hunger, lack of sleep, citrus fruits, some cheeses, and perfumes) and its

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deactivators (sleep, pregnancy, triptans) [2,3,5,13,14,15,16,17,18].

The word migraine was derived from the Latin word "hemicrania" meaning "half" (Hemi) "skull" (crania) [19]. This term was first used by Galenus of Pergamon, a Greek physician, surgeon, and philosopher in the Roman Empire, to describe the pain felt across one side of the head during a migraine. He also suggested that the pain originated in the meninges and vasculature of the head. In addition, he pointed towards a connection between the stomach and the brain due to the vomiting that seemed to be related to migraines [19,20].

The mainstay of pharmacologic therapy is the judicious use of one or more of the many drugs that are effective in migraines [3,4,5,8,11,13,21,22]. The selection of the optional regimens of migraine patients depends on several factors, the most important of which is the severity of the attack (table 1) [2,3]

The occurrence of three or more attacks per month is an indication for prophylactic treatment. [3,4,6] The criteria for preferring one prophylactic drug to another are based upon: [14]

- Evidence of efficacy [4,8,23]
- Comorbidity and the anticipated effect of the drug upon it [2,3,4,23]
- Contraindications including risk in pregnancy [5,23]
- Good evidence that poor compliance is a major factor in impairing the efficacy of migraine prophylaxis and that once-daily dosing is preferable [23]

The formal evidence – base for efficacy is good for beta-blockers and adequate for amitriptyline [3,4,6,21,23]

Other drugs include cyproheptadine, sodium valproate, gabapentin, methysergide & verapamil can be used [3,23]

Table (1) classification of migraine according to the severity [24] Note NSAIDs, non-steroidal anti-inflammatory drugs 5HT, 5 hydroxytryptamine

Stage	Diagnosis	Therapies
Mild migraine	Occasional throbbing headaches No major impairment of functioning	NSAIDs
Moderate migraine	Moderate or severe headaches Nausea common Some impairment of functioning	Combination analgesics, oral 5HT, agonists Oral, nasal or SC5HT, agonists Oral dopamine antagonists
Severe migraine	Severe headaches >3 times per month Significant functional impairment Marked nausea and/or vomiting	SC, IM, or IV5HT$_1$ agonists IM or IV dopamine antagonists Prophylactic medications

Methods:

This prospective clinical study was performed on 80 patients with severe migraines among those who attended the neurologic consultation in Al-Hussein general hospital in Karbala which is affiliated with The University of Al-Ameed. The study started in January 2007; patient admittance was completed in October 2007. Subjects were considered potentially eligible if they were at least 16 years old; were in good general health; and not pregnant.

The efficacy and **tolerability** in this study was assessed as rapid and consistent freedom from pain and other symptoms, return to normal function, minimal need for repeat dosing, and minimal adverse events. If this happens, it is considered as a **good response**.

For each included patient a detailed history was taken including name, age, sex, residence, marital status,

aggravating factors (food, stress) history of contraceptive pills, menstruation, and family history (regarding migraine) (Data not shown).

Statistical methods: - student paired t-test (for dependent data), student unpaired t-test (for independent data), or chi-square (x^2) were used accordingly to assess whether the obtained differences could be accepted as insignificant (if $p > 0.05$) or significant if ($0.01 \leq p \leq 0.05$) or highly significant if ($p < 0.01$).

First Part of the study

The enrolled patients were randomly assigned into 2 treatment groups, for each group the follow up duration was 10 days: -

1. Single oral analgesic group (n=40). Patients of this group received a common medication of treating migraine attack which is diclofenac, the dose is 50 mg tablet twice day when needed which is the usual dose of diclofenac. [25]

2. Combined oral analgesics (Excedrin) n=40. Patients of this group received a tablet that consists of (aspirin 250 mg + acetaminophen 250 mg + caffeine 65 mg) this tablet is commercially called Excedrin which was given four times/day when needed as a maximus dose [26] and the patient is informed about the possible side effects.

Both these two groups were given additionally pizotifen tablet 0.5 mg twice daily as a prophylactic drug because all enrolled patients were suffering from severe migraine which necessitates a prophylactic medication [27,28,29]. Pizotifen is available in the market and not expensive for that reason this medication was chosen.

Second Part of the study

The 2 groups from the first part of the study were followed up to see the response to the prophylactic treatment regimen. The patients who showed good response in the **first part (n=46)** out of the two groups were divided randomly into **three groups** with different prophylactic regimens: -

1. Oral **pizotifen** 0.5 mg tablet twice daily, **n=15**.

2. Oral **pizotifen** 0.5 mg tablet twice daily + **propranolol** 20 mg tablet twice daily, **n=15**.

3. Oral **pizotifen** 0.5 mg tablet twice daily + **amitriptyline** 10 mg only at night, **n=16**.

The enrolled patients were followed up for the **next four months** to be seen at each month during the follow-up period to evaluate the efficacy of these prophylactic regimens in preventing the recurrence of the attack of the migraine. However, each patient was advised to use Excedrin or diclofenac tablet when needed as abortive to migraine attack.

A preventive migraine drug is considered successful if it reduces migraine attack frequency or days by at least 50% within 3 months. [30]

RESULTS:

Subject population characteristics

Of the 80 subjects enrolled in the study, 64 (80%) were aged 16-40 years with a mean age of 25.33 ± 0.8 years while only 16 (20%) patients were older than 40 years with a mean age of 44.0 ± 0.7 years. The sex distribution shows 68 (85%) patients were females while 12 (15%) patients were males (Table 1).

The efficacy of single versus combined analgesics in the treatment of severe migraine

The percentage of patients who showed good response to the therapy (good response means rapid and consistent freedom from pain and other symptoms, return to normal function, minimal need for repeat dosing, and minimal adverse events) was significantly higher in the Excedrin group (85%). While only (30%) of patients showed a good response with diclofenac therapy ($p \leq 0.01$) (Table 3).

The response of the enrolled patients to the different prophylactic regimens used

There was a significant difference ($p \leq 0.05$) between the pizotifen alone group and pizotifen + propranolol

group, where 60% showed good response with pizotifen + propranolol and 33.3% showed good response with pizotifen only.

On the other hand, the difference between the pizotifen alone group and pizotifen + amitriptyline group was found to be highly significant p≤0.01. 87.5% showed good response with pizotifen +amitriptyline group (Table 4).

All of these prophylactic regimens were well tolerated along the trial period (i.e., 4 months) without noticeable side effects among the included patients in this prospective study apart from sedation noticed with the pizotifen +amitriptyline group.

Table (2): Demographics of patients

Age of patients	Number of patients	Sex	
		Male	Female
16 – 40 y	64	8	54
More than 40 y	16	4	12
Total number and percentage	80 100%	12 15%	68 85%

Table (3): Distribution of patients according to therapy-induced improvement in diclofenac and Excedrin groups.
^{H5} Highly Significant difference (P≤ 0.01)

Treatment group	Number of patients	Number of patients with good response	Percentage
Diclofenac	40	12	30%
Excedrin	40	34^{HS}	85%

Table (4): Patients response according to different prophylactic treatment regimens

^{S}: Significant difference (P<0.05) when compared with the pizotifen group

^{H5}: Highly Significant difference (P≤ 0.01) when compared with pizotifen group

Treatment group	No. of patient	No. of the patient with good response	Percentage
pizotifen	15	5	33.3%
Pizotifen + propranolol	15	9^{s}	60%
Pizotifen + amitriptyline	16	14^{HS}	87.5%

DISCUSSION:

Migraine is a common neurological disease that causes a variety of symptoms, being headache the hallmark [3,15,16,28]. It can interfere significantly with the patient's life and severe attacks can lead to significant functional impairment [3,10,13,30,31]. Its aetiology is largely unknown. [15,16,28]

Although migraine can occur in all ages, it usually begins in adolescence. In more than 80% of patients, the onset is before 30 years of age and the frequency of the attacks largely decreases in elderlies [3,15,16]

In this prospective study, 80% of the included patients have aged 16-40 yrs. with a mean age of 25.3 years, while only 20% of the patients were aged above 40 years which is similar to other studies [3,15,16]. Migraine is three times more common in women than men. [28,31] In the present study, the ratio of females to males was 5.5:1 which can be because this study deals with severe migraine only which is more common among female patients. [31]

Patients without contraindications should be offered

acute therapy for migraine, starting with NSAIDs. Those who do not respond after appropriate trial periods should be offered another therapy. [4,27,28,29] Drug treatment should be selected for each patient according to his or her need. [27,28,29]

There is good quality of evidence supporting the use of Non-Steroidal Anti-inflammatory drugs (NSAIDs) such as acetaminophen, diclofenac and acetylsalicylic acid which can reduce both the severity and the duration of migraine attacks significantly [5,9,14]. Non-Steroidal Anti-inflammatory drugs (NSAIDs) are most effective when given early in migraine attack [5,9,14,28]. Diclofenac-potassium 50-100 mg in non-delayed-release with or without intramuscular injection can be used for the treatment of migraine. [5,9,19]

Most migraine headaches respond to analgesics such as paracetamol [5,8] or aspirin [5,8,9,22] but because intestinal peristalsis is often reduced during migraine attack the medication may not be sufficiently well absorbed to be effective. [5,8,22] Caffeine is thought to enhance analgesics absorption and possibly to have a vasoconstrictor activity. [7] Excedrin is a combination of paracetamol, aspirin and the therapeutically active caffeine approved by FDA to treat all of the symptoms of migraine. NSAIDs offer several advantages over prescription drugs, including easy access, lower cost, and fewer adverse effects [8,22]

In the first part of the present study, a comparison was made between 10 days course of therapy with either diclofenac with pizotifen for 40 patients or Excedrin with pizotifen on another 40 patients suffering from severe migraine. The percentage of patients with good response to treatment with either Excedrin or diclofenac were 85% and 30% respectively and the difference between the two treatment groups was highly significant ($P \leq 0.01$).

Excedrin was well tolerated by the patients during the 10 days of the therapy apart from slight gastric irritation experienced by some. The studied patients showed:

- A noticeable reduction in pain within 30 minutes after treatment initiation.
- Major improvement in their ability to take part in normal daily activities (at an affordable price).

These results indicate that it is worth trying to prescribe Excedrin for the management of acute migraine attacks before prescribing more expensive drugs with the potential for severe side effects and drug interaction.

The second part of the study dealt with the prophylactic treatment of migraine. Whereas the goal of acute therapy is to abort a migraine attack once it has started, the goals of prophylactic treatment are to prevent attacks, thereby reducing headache frequency, severity and associated disability and decreasing reliance on acute treatment, which may be contributing to concurrent medication over use headache [25].

The occurrence of three or more severe attacks per month is an indication for prophylactic treatment [4,27,28,29,30,31]. These drugs must be taken daily and there is usually a lag of 2-6 weeks before an effect is seen and they should be continued for 4-6 months [4,27] Pizotifen has been widely used for many years but clinical trials evidence of its efficacy is limited [4,29], however in our hospital, pizotifen is available and free for patients in the hospital and for that reason it was chosen to be given to all patients. Beta-blockers are considered the first line if not contraindicated. Of these, propranolol (40-240mg) is the most evidence-based and widely used drug. [4,29]. Antidepressants, amitriptyline 10-75 mg the most widely prescribed, are considered the first line when migraine coexists with trouble some tension-type headache, another chronic pain condition, disturbed sleep or depression. However, antidepressants can be used to prevent migraine even if there is no underlying depression [4,6,23].

Although many patients benefit from these therapies, studies have shown that patient adherence to existing oral preventives is low, often because of suboptimal efficacy and poor tolerability. There is still unmet need for more effective, better tolerated prophylactic therapies [23].

We have demonstrated that the use of combination prophylactics is superior to single agent in the prevention of migraine attacks. While only 33.3% of the patients showed good response with pizotifen alone, 60% showed good response when pizotifen combined with propranolol. Nevertheless, interestingly 87.5% of the patients showed good response with the combination of pizotifen and amitriptyline. The difference was significant between pizotifen alone and pizotifen and propranolol groups ($P \leq 0.05$) and highly significant between the first group and the pizotifen+ amitriptyline group. A synergistic effect might explain the improved efficacy of the combination of prophylactic medications in this study. Our observations parallel, to some extent, those in a report by Rao and colleagues in which they demonstrated that the combination of propranolol and cyproheptadine is more efficacious than propranolol alone in the prevention of migraine. [33, 34,35]

Further investigations are necessary to assess whether using the maximum dose of these prophylactic agents would result in any better outcomes.

CONCLUSION

1. Combined analgesic, Excedrin, is better than the single analgesic, diclofenac, in the treatment of migraine headache attacks.

2. Combined prophylactic drugs (amitriptyline or propranolol with pizotifen) are better than pizotifen alone in the prophylaxis of severe migraine.

Study limitations:

1. Sample size.
2. Single-center study.
3. Needs more duration for follow-up.

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Conflict of interests

The authors declare no conflicts of interest.

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Data availability

The data analyzed during this study are available from the corresponding author upon request.

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فعالية وتحمل استخدام الأدوية المركبة مقابل المسكنات الفردية والأدوية الوقائية في حالات الصداع النصفي الشديد

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ملخص

الخلفية: الصداع النصفي هو سبب شائع للصداع الأولي الذي قد يتداخل مع الأنشطة اليومية العادية التي يتم استخدام طرق مختلفة من العلاج لها. في الدراسة الحالية، تم تقييم أنواع مختلفة من المسكنات والأدوية الوقائية من حيث فعاليتها وتحملها.

الهدف: تقييم الفعالية والتحمل لما يلي:

1. مسكن مشترك، إكسدرين (أسبرين مع باراسيتامول وكافيين) بالمقارنة مع مسكن واحد، ديكلوفيناك، كعلاج لنوبة الصداع النصفي الشديدة.

2. الجمع بين الأدوية الوقائية، أميتريبتيلين، أو بروبرانولول مع البيزوتيفين بالمقارنة مع البيزوتيفين وحده كأدوية وقائية للصداع النصفي الشديد.

المواد والطرق: الجزء (1): تم تسجيل 80 مريضًا يعانون من الصداع النصفي الشديد وتعيينهم عشوائيًا لتلقي قرص فموي إما من Excedrin الأسبرين 250 ملغم، والأسيتامينوفين 250 ملغم، والكافيين 65 ملغم) أربع مرات يوميًا عند الحاجة أو ديكلوفيناك 50 ملغم مرتين يوميًا عند الحاجة كعلاج لنوبة الصداع النصفي، بالإضافة إلى استخدام البيزوتيفين مرتين في اليوم في كلا المجموعتين الفرعيتين لمدة 10 أيام. الجزء (2): تم تقسيم 46 مريضًا ممن أظهروا استجابة جيدة من الجزء الأول من الدراسة بشكل عشوائي إلى ثلاث مجموعات فرعية مع أنظمة وقائية مختلفة.

1. قرص pizotifen 0.5 ملغم عن طريق الفم مرتين في اليوم، (16 مريضا).

2. pizotifen عن طريق الفم 0.5 ملغم مرتين في اليوم مع بروبرانولول 20 ملغ قرص مرتين في اليوم، (15 مريضا).

3. قرص بيزوتيفين 0.5 ملغم مرتين يومياً مع أميتريبتيلين 10 ملغم قرص مرة واحدة ليلاً. (15 مريضا). تم تقييم الفعالية من خلال تحديد عدد المرضى الذين يظهرون تحسنًا مع عدم وجود صداع أو صداع خفيف. معنى التحمل في هذا البحث هو عدم ظهور آثار جانبية او القليل منها .

النتائج: الجزء (1): تم الحصول على استجابة جيدة للعلاج في 30٪ من مجموعة الديكلوفيناك مقابل 85٪ من مجموعة الاكسدرين.($p<0.01$). الجزء (2): تم الحصول على استجابة جيدة للعلاج في 33.3٪ من مجموعة البيزوتيفين مقابل 60٪ من البيزوتيفين مع مجموعة البروبرانولول ($P< 0.05$) مقابل 87.5٪ من البيزوتيفين مع مجموعة الأميتريبتيلين .($p<0.01$)

الخلاصة: الأدوية المركبة أكثر فعالية من الأدوية المنفردة في علاج الصداع النصفي الشديد والوقاية منه.

الكلمات الدالة: إكسيدرين، ديكلوفيناك، أميتريبتيلين، بروبرانولول، بيزوتيفين، صداع نصفي شديد.

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The Effectiveness of Zodia Leaves (Evodia Suaveolens Scheff) Oil as *Aedes aegypti* L Mosquito Repellent in Papua

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ABSTRACT

Dengue hemorrhagic fever (DHF) is a disease caused by infection with the dengue virus. It is controlled by using repellents that protect humans from mosquito bites. One of the repellents used by the community includes DEET chemicals but natural repellents used against mosquitoes such as the zodia plant are also needed. Zodia leaves (*Evodia suaveolens* Scheff) contain linalool and -pinene compounds as well as evodiamine and rutacarpine. Linalool functions by disrupting the nervous system in mosquitoes thereby causing convulsion and death. Therefore, this study aims to formulate and evaluate the zodia oil and test its effectiveness as a mosquito repellent (*Aedes aegypti* L) with repelling power method. The formulations were prepared with various concentrations of 25 %, 50 %, and 75 %. Based on the results, the protective power of Formula I (25 %) for 0, 1st, 2nd, and 3rd hours, respectively, was 100%, 100 %, 89.28 %, and 92.85 %. Furthermore, Formula II (50 % concentration) showed a protective power of 100 %, 100 %, 90.90 % and 91.66 %, while the third Formula (75 % concentration) showed a protection power of 100 %, 100 %, 96.15% and 93.33%. Therefore, it was concluded that the three zodia oil formulas are effective as a repellent against *Aedes aegypti* L mosquitoes.

Keywords: *Aedes aegypti* L., Dengue hemorrhagic fever, *Evodia suaveolens* Scheff, Repellent, Zodia oil.

I. INTRODUCTION

Dengue hemorrhagic fever (DHF) is a disease caused by infection with the dengue virus which is transmitted into the human body through the *Aedes aegypti* L mosquito. All regions in Indonesia are at risk for contracting this disease because both the pathogen and the vector are widespread in residential and public areas except in places with an altitude of 100 m above sea level. Therefore, this disease is still a public health problem and is endemic in some districts/cities in Indonesia [1]. The use of drugs and chemicals such as DEET **(**N**,** N-Diethyl-meta-toluamide) **to** eradicate *Aedes aegypti* L mosquitoes often causes side effects hence, this increased the public interest in using medicinal plants that have long been used by the predecessors such as zodia [2].

Zodia (*Evodia suaveolens* Scheff) is an endemic plant to Papua used traditionally by the local people [3], it is used as a mosquito repellent, body odor remover, as well as wound and toothache medicine [2]. Furthermore, zodia belongs to the Rutaceae family which contains evodiamine. According to the results of a gas chromatographic analysis conducted at the Research Institute for Spices and Medicinal Plants (Balittro), the oil distilled from the leaves of this plant contains linalool (46%) and a-pinene (13.26 %) where linalool is well known as a mosquito repellent [4-6].

Previous studies on zodia related to the pharmaceutical preparations have been carried out, such as the potential of zodia leaf essential oil (*Evodia suaveolens* Scheff) as an

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insecticide for the *Aedes aegypti* L mosquito using the electric method [7], the effectiveness of zodia leaf essential oil (*Evodia suaveolens* Scheff) lotion repellent against *Aedes aegypti* L mosquito [8], and zodia soap as a repellent against *Aedes aegypti* L mosquitoes [9]. The studies also include the protective power of liquid soap repellent mosquito of zodia leaf essential oil (*Evodia suaveolens* Scheff) against *Aedes aegypti* L mosquito [10] and the effectiveness of repellent lotion preparations with a combination of zodia leaves (*Evodia suaveolens* Scheff) and lemongrass essential oil (*Cymbopogon citratus*) against *Aedes aegypti* L mosquitoes [11]. However, products in the form of oil have not been made.

II. MATERIAL AND METHODS

Material preparation

Zodia leaf samples (*Evodia suaveolens* Scheff) were obtained from a plant garden on Kertosari Street, Sabronsari Village, West Sentani District, Jayapura, Papua. The already dark green plants were prepared at the age of 1 and then separated from the stems using a knife. The zodia leaves were then cleaned using running water, dried in the open air, and not exposed to direct sunlight for 1 day to 2 days to reduce the water content in the samples [12].

Zodia leaf essential oil

Zodia leaf essential oil was distilled using the steam distillation method for 4 hours, the leaves (1 kg) were dried in the open air and not exposed to direct sunlight. The essential oil was separated from the water using a separating funnel and then anhydrous Natriumsulphate (Na_2SO_4). The final product was obtained and stored in a closed vial bottle in a refrigerator [12].

Zodia leaves contain linalool and alpha-pinene up to 46% and 13.26% respectively. Linalool (3,7-dimethyl-1,6-octadien-3-ol) is a contact poison that increases sensory nerve activity in insects, furthermore, it causes motor nerve stimulation, causing seizures and paralysis in some insects [4]. After the wet zodia leaf samples were taken, the results obtained after aeration was 6 kg (40%) of the dry samples.

Characterization of zodia leaf essential oil

The zodia plant contains essential oils that have the characteristics of linalool compounds which was obtained using preparative Thin Layer Chromatography (TLC) (silica Gel $60F_{254}$) with toluene: ethyl acetate (93: 7) as eluent, detection using 10 % Sulfuric acid (H_2SO_4) reagent, and lavender essential oil as a comparison. A positive test result is indicated when the sample produces a blue color and the Rf value = 0.3 [13].

Preparation of zodia oil

Zodia oil formula was divided into three concentrations, namely 25%, 50%, and 75%, while one formula was used as a placebo. The selection of different concentrations was carried out to determine the significant difference between the three concentrations of zodia essential oil as a mosquito repellent. In contrast, the positive control used mosquito repellent sold in the market while hands with no test sample were used as a negative control.

Table 1: Zodia oil formula composition

Material	Formula (%) (v/v)		
	I	II	III
Zodia oil (mL)	25	50	75
Coconut oil(mL)	75	50	25
Total	100	100	100

The zodia oil preparations were made by mixing materials, for example, coconut oil as a carrier or solvent into a volumetric flask and then added with zodia leaf essential oil. The mixture was homogenized, poured into a 20 mL container and the composition of each oil in the container (zodia essential oil: coconut oil) is 1: 3, 2: 2, and 3: 1 (Table 1). Coconut oil functions as a carrier oil or solvent [14].

Zodia leaves (Figure 1-a) contain linalool in the essential oil which kills mosquitoes due to its function in increasing

sensory nerve activities and stimulation of motor nerves, thereby causing mosquitoes to experience paralysis and death. The essential oil was extracted using the steam distillation method due to its high volatility, such that when exposed to hot steam, it produces a distillate. Furthermore, the essential oil was obtained and separated from the water using a separatory funnel, and then with anhydrous Na_2SO_4 to bind and separate the water from the essential oil. From the sample, ± 6 kg produced 17.18 mL of essential oil hence, the average essential oil obtained was 2.86 mL/kg. The results of the organoleptic examination were in the form of a liquid-like oil, yellow with a characteristic zodia odor, and a pH of 6 (Figure 1-b).

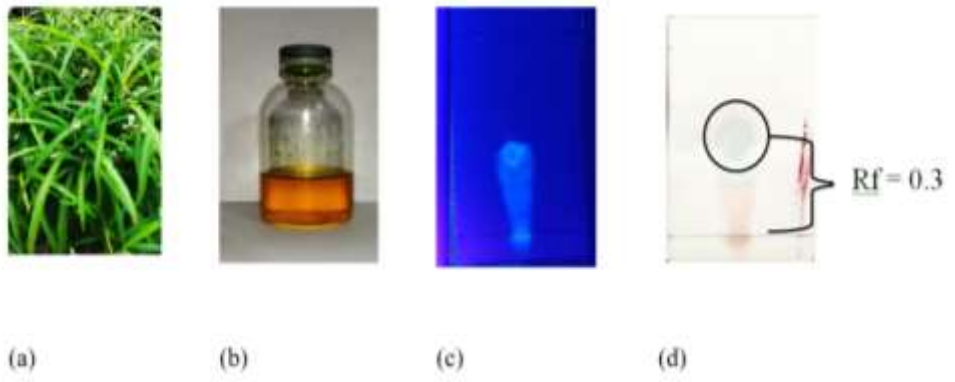


Figure 1: Zodia leaves (a); zodia essential oil from steam distillation (b); TLC test results of zodia leaf essential oil with eluent toluene: ethyl acetate 97:3), before spraying H_2SO_4 10% TLC under UV lamp 254 (c), after spraying H_2SO_4 10% (d)

The TLC test was carried out using a plate containing $60F_{254}$ silica gel to determine the presence of linalool contained in the essential oil of zodia leaves. Furthermore, the solvent used was toluene: ethyl acetate (97: 3 v/v) while 93% toluene was used as the eluent, given that it is a non-polar solvent and was adjusted to the character of the nonpolar zodia leaf essential oil. Meanwhile, 3% ethyl acetate was added to reduce the level of the polarity of the eluent. Based on the calculated Rf value and the color of the stain compared with the data in the literature, a positive test for Linalool is indicated by a blue color and Rf value = 0.34 (Figure 1-c, d).

Collection of Aedes aegypti L mosquitoes

The mosquitoes were collected at 08.00-10.00 by volunteers wearing black clothes and recheck under the microscope to ensure the mosquitos was *Aedes aegypti*. When a mosquito approaches a volunteer, it is caught with a suction device that has been specially prepared and placed into a glass cage.

Research Design

The research design used is the evaluation of zodia oil preparations.

a. Organoleptic test includes appearance including shape, color, and the smell of zodia oil (Yuniarsih, 2010).
b. The Power of Hydrogen (pH) Acidity Test was carried out by weighing 1 gram of zodia oil and then dipping the pH paper into the solution. The degree of acidity (pH) obtained was observed and recorded [15].
c. Patch Test
d. The safety test was carried out on volunteers for 15 minutes, the reaction was then observed to determine the occurrence of irritation/allergy [15].
e. Test of Protection as Repellent

This research was conducted and involved ethical number 01/KEPK-JYP/VII/2020. The lower left arm was smeared with repellent material, namely zodia oil, while the lower right arm was used as a negative control [16]. Hands that have been sprayed with zodia oil were left for 5 minutes, placed in the mosquito cage for 15 minutes, and then removed and placed back after 1, 2, 3, 4, 5, and 6 hours with an observation period of 15 minutes every hour to determine the protection power.

The effectiveness test of Zodia oil was carried out in a mosquito cage measuring 10 x 10 x 10 cm, the walls were made of glass and covered with nylon gauze. 3 cages were provided for testing negative control (-), positive control (+), and placebo [4-6, 10]. Each was placed in a sample of 15 *Aedes aegypti* L mosquitoes which has not sucked blood at all. The arm was sprayed with Zodia oil of each test formula as well as for the control (-) and (+).

The power of protection against mosquito disturbances was determined by the formula:

$$Pp = \frac{C-T}{C} \; X \; 100\ \%$$

Where:

Pp: Protective power

C: Numbers perched on control arm (does not contain zodia leaf oil)

T: Figures perched on arms smeared with zodia leaf oil

III. RESULTS AND DISCUSSION

Zodia oil preparation

In the formulation of zodia oil preparations, the results showed three variations namely coconut oil F I (25%: 75%), FII (50%: 50%), and FIII (75%: 25%) (Figure 2) which indicates that the formula was well formulated and homogeneous. Both of the materials were mixed and then shaken until homogeneous as observed from the absence of color separation in the two oils. Also, the volatile oil which was previously yellow became faded because it has been mixed with the coconut oil base.

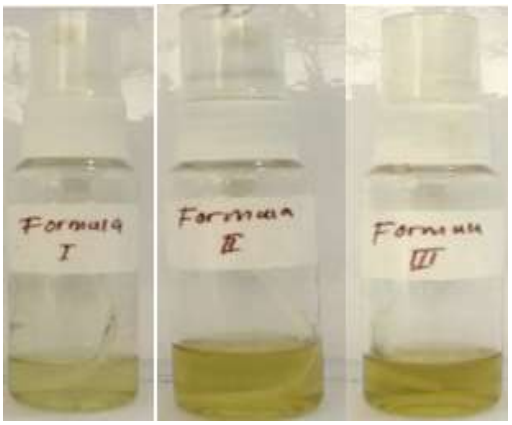


Figure 2: Preparation of zodia oil concentration of 25% (a), 50% (b), 75% (c).

Evaluation of Zodia oil Preparations

The organoleptic examination showed that F I had a faint yellow color and a characteristic smell of zodia oil, while F II and III had a faded yellow color and a very pungent smell of zodia oil due to variations in concentrations of 50% and 75% (**Table 2**).

Table 2: Organoleptic Results of Zodia Oils

Formula	Organoleptic Observation		
	Form	Color	Smell
Formula I	Liquid	Fade Yellow	Zodia
Formula II	Liquid	Yellow	Typical Zodia Sting
Formula III	Liquid	Yellow	Typical Zodia Sting

The average pH for formulas I, II, and III was 5, 5, and 5.3 due to several factors such as the concentration of zodia oil of 75% where the pH was 6-7 [7], and the 25% coconut oil concentration with pH of 5 hence, the pH in the first test in formula III was 6, meanwhile, in the second and third tests, the pH was 5 (Table 3).

Table 3: Zodia oil pH test results

Formula	Average of pH
Formula I	5
Formula II	5
Formula III	5.3
Placebo	5
Positive control	5

Note: Formula I concentration of zodia essential oil (25%), Formula II concentration of zodia essential oil (50%), Formula III concentration of zodia essential oil (75%), and Placebo (coconut oil).

The placebo and the positive control both had a pH of 5. The pH value of the three zodia oil formulas is applicable because it is in line with the physiological pH range of the skin namely 4.5-6.5. When pH is too alkaline, it causes scaly skin, meanwhile, when the pH is too acidic, it causes skin irritation [16].

The safety of the preparations was tested using a patch test on 20 volunteers sprayed with zodia oil and a placebo on the back of the hand for 15 minutes. Two volunteers in formulas II and III experienced allergies in the form of itching on the hands along with redness but there was no swelling which is presumably caused by different skin types in each volunteer (Table 4). Previous studies showed that there are different types of skin namely normal, dry, and oily [16-18]. When the pH is too alkaline, it leads to dry and sensitive skin, in contrast, when the pH is too acidic it causes skin irritation.

Table 4: Zodia oil safety test

Formula	Criteria			Total panels
	No irritating	Slightly irritating	Irritating	
Formula I	20	-	-	20
Formula II	19	1	-	20
Formula III	19	1	-	20
Placebo	20	-	-	20

Note:

- No irritating Does not cause redness, does not itch and does not swell
- Slightly irritating Redness, itching, and no swelling
- Irritating Redness, itching, and swelling

Test of zodia oil protection power as a repellent for Aedes aegypti L . mosquitoes

The mosquitoes were collected between 08.00-10.00 given that the blood-sucking behavior of female *Aedes aegypti* L mosquitoes occurs every two to three days in the morning until the afternoon, namely 08.00-12.00 and 15.00-17.00. Female mosquitoes often bite more than one person (multiple bitter) to get enough blood. Furthermore, disease transmission occurs because every time a mosquito sucks blood, it transfers the saliva through its proboscis[19].

Adult *Aedes aegypti* has a medium size with brownish-black body color. The body and legs are covered with scales with silvery-white stripes, meanwhile, on the posterior part of the body, there are two vertically curved lines, namely the left and the right, which are characteristics of this species. In general, the scales fall out or fall off easily, hence, it is difficult to identify older mosquitoes. The size and color of *Aedes aegypti* differ between populations, depending on environmental conditions and also the nutrients obtained during the development period [20].

In this study, the essential oil was placed in a bottle and was applied by spraying [26]. The advantage of spray mosquito repellent compared to other types includes it is easier to manufacture and practical, does not cause air pollution, and saves electricity. In addition, mosquito repellent spray tends to reach hidden places such as under the bed, behind window curtains, and hung cloths [12].

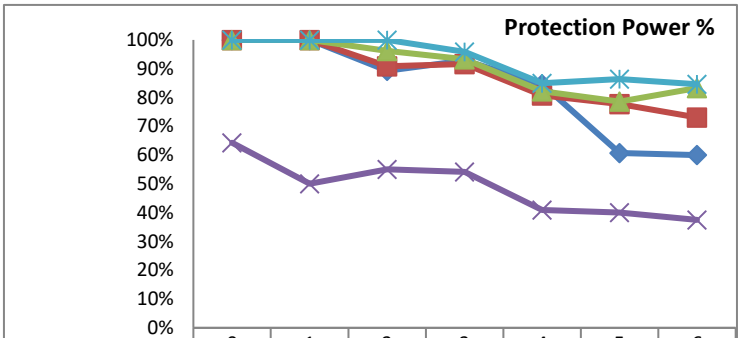
The effectiveness of zodia oil as a repellent in this study was tested in three cages made of glass and covered with nylon gauze where each contained 15 *Aedes aegypti* L mosquitoes that had not sucked blood. This test was carried out every 15 minutes for 6 hours, observations were made on the protective power of each zodia oil formula, placebo, and positive control against *Aedes aegypti* L mosquitoes.

The graph in Table 5 shows that the higher the concentration of zodia oil, the greater the protective power against mosquitoes, and the higher the time, the lower the protective power. Furthermore, the protective power was observed to decrease in the 3rd to 6th hour but not as drastically in formulas II and III. The decrease in the repellent effect was due to the zodia leaf essential oil in preparation. The sweat from the volunteers' hands removed the essential oil from the skin surface.

The mechanism of action of essential oils as a mosquito repellent is by releasing odors and repellent compounds. Meanwhile, the human skin secretes lactic acid and other excretory products that mosquitoes use to detect human odors and presence. Essential oils when applied to human skin are absorbed into the pores of the skin and evaporate in the presence of body heat, producing an odor that is detected by the mosquito's chemical receptors [21-22]

In the mosquito's anatomy, the antennae and palps function as chemical senses that are very sensitive and stimulated by chemical odors. When the essential oil evaporates, the odor released is detected by the chemoreceptors which then triggers nerve impulses. This smell confuses mosquitoes hence, the brain responds to avoid the smell. Essential oils also work by masking odors in humans, hence, the receptors on the senses are disturbed and mosquitoes are unable to detect chemical products from humans [23].

Table 5: Zodia oil protection test as a repellent for *Aedes aegypti* L. mosquitoes



	0	1	2	3	4	5	6
F I (25%)	100.00%	100.00%	89.28%	92.85%	84.61%	60.71%	60.00%
F II (50%)	100.00%	100.00%	90.90%	91.66%	80.76%	77.77%	73.07%
F III (75%)	100.00%	100.00%	96.15%	93.33%	82.13%	78.57%	83.33%
PLASEBO	64.28%	50.00%	55.00%	54.16%	40.90%	40%	37%
KP	100.00%	100.00%	100.00%	95.83%	85.00%	86.35%	85%

F I: Formula I (Zodia leaf essential oil 25: 75 coconut oil)
F II: Formula II (Zodia leaf essential oil 50: 50 coconut oil)
F III: Formula III (Zodia leaf essential oil 75: 25 coconut oil)
Placebo: Base Control (coconut oil)
KP: Positive Control (repellent on the market)

Furthermore, the graph in Table 5, shows that the higher the concentration of the essential oil, the greater the protective power. Also, the higher the time, the smaller the protective power of the mosquito repellant. The repellency power decreased because essential oils are volatile hence, the effect was reduced gradually. The evaporation process is influenced by environmental and human factors. Environmental factors include room temperature, wind, and humidity, while human factors include body temperature and activities carried out by the volunteers during testing.

Based on the results, the repellency of zodia oil is effective, this is in line with a study where, several preparations were made and had >80% protection results such as the use of solid soap of zodia leaves essential oil as a repellent against *Aedes aegypti* L mosquitoes with a concentration of 1.5% which provided a protective power of 80.93% at 0 hours [9]. Another study also tested the effectiveness of repellent lotion of a combination of zodia leaves (*Evodia suaveolens* Scheff) and lemongrass essential oil (*Cymbopogon citratus*) against *Aedes aegypti* L mosquitoes [11] which had 100% protective power at 0 hours. Also, it was found that the effectiveness of 1.5% zodia leaf essential oil lotion against *Aedes aegypti* mosquitoes had a protection power of 88.07% at 0 hours [8]. Another study showed that the potential of zodia leaves essential oil (*Evodia suaveolens* Scheff) as an insecticide for *Aedes aegypti* L by the electric method [7] had protective power of 100% within 20-30 minutes.

Table 5 shows that the positive control had higher mosquito repellency than zodia oil at concentrations of 25%, 50%, and 75% because there was no chemical substance to maintain repellency at higher preparations. Meanwhile, the active chemical contained in the citrus peel scent is DEET with a more concentrated 130 g/L content which functions to inhibit mosquitoes for longer. Most of the mosquito repellents on the market contain the active ingredients diethyltoluamide (DEET), dichlorophenyl dimethyl phosphate (DDP), Malathion, and Parathion. DEET works by inhibiting the chemical receptors of carbon dioxide and lactic acid in mosquitoes.

Compared with the positive control, the zodia oil formulations showed a significant difference, while the positive control showed better results. The positive control used DEET (diethyl-meta toluamide), which is more effective in repelling mosquitoes than the administration of essential oil in zodia oil. DEET functions by manipulating the smell and taste produced from the skin and inhibiting the receptors on the mosquito antennae to prevent the detection of the skin. However, there are several side effects of the DEET, for example, it is not suitable for breastfeeding mothers and children below 2 months of age. Also, in high doses and for a long time, it causes skin irritation, erythema (redness of the skin), muscle cramps, and rashes. In addition, repeated use and prolonged absorption through the skin potentially lead to poisoning especially in children [24, 27].

The continuous use of these chemicals, in addition to hurting human health, also makes mosquitoes resistant [25]. Based on the government regulations through the Pesticide Commission of the Ministry of Agriculture (1995) a repellent preparation is said to be effective when it has a protective power of >90% and lasts for 6 hours of observation. The results of this study indicated that zodia oil with an essential oil concentration of 25% protects up to >90% at the 0 to 1 hour at 100% and the 3rd hour at 92.85%, while 50% protect up to >90% at 0 to 1 hour by 100%, and the 2nd hour by 90.90% and at the 3rd hour by 91.66%. Furthermore, the zodia oil with a concentration of 75% protects up to >90% at the 0 to 1 hour at 100%, 2nd hour at 96.15%, and the 3rd hour at 93.33% (Table 5). Formula III with a concentration of 75% at the 6th hour had higher protective power because this study involved living things which led to the occurrence of range errors and other factors, namely the condition of mosquitoes on the surface of volunteers' hands due to the effect of the repellent in the previous 5 hours. Based on these results, no concentration of zodia leaf essential oil lasted

effectively for 6 hours, hence, it was concluded that the zodia oil formula as a repellent has not been maximized to repel mosquitoes for more than 6 hours, due to the absence of active ingredients such as diethyltoluamide (DEET), dichlorovinyl dimethyl phosphate (DDP), malathion, and parathion in maintaining dissipation time.

CONCLUSIONS

Zodia leaf essential oil is applicable as an effective mosquito repellent though the positive control is higher than the test sample. From the experiment, the three zodia oil formulas with concentrations of 25%, 50%, and 75% are effective as a repellent against the *Aedes aegypti* L mosquito.

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فعالية زيت أوراق الزوديا (Evodia Suaveolens Scheff) مثل Aedes aegypti L طارد البعوض في بابوا

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ملخص

حمى الضنك النزفية (DHF) هي مرض تسببه الإصابة بفيروس حمى الضنك. يتم التحكم فيه عن طريق استخدام المواد الطاردة للحشرات التي تحمي الإنسان من لدغات البعوض. أحد المواد الطاردة للحشرات التي يستخدمها المجتمع تشمل المواد الكيميائية DEET ولكن هناك حاجة أيضًا إلى المواد الطاردة الطبيعية المستخدمة ضد البعوض مثل نبات الزوديا. تحتوي أوراق Zodia (Evodia suaveolens Scheff) على مركبات linalool و –pinene بالإضافة إلى evodiamine و .rutacarpine يعمل اللينالول عن طريق تعطيل الجهاز العصبي في البعوض مما يسبب التشنج والموت. لذلك ، تهدف هذه الدراسة إلى صياغة وتقييم زيت الزوديا واختبار فعاليته كطارد للبعوض (Aedes aegypti L) مع قوة طاردة. تم تحضير المستحضرات بتركيزات مختلفة 25٪ ، 50٪ ، 75٪. بناءً على النتائج ، كانت القوة الوقائية للصيغة I 25٪ للساعات 0 و 1 و 2 و 3 على التوالي 100٪ و 100٪ و 89.28٪ و 92.85٪. علاوة على ذلك ، أظهرت الصيغة II تركيز 50٪ قوة وقائية بنسبة 100٪ و 100٪ و 90.90٪ و 91.66٪ ، بينما أظهرت الصيغة الثالثة (تركيز 75٪) قوة حماية 100٪ و 100٪ و 96.15٪ و 93.33 ٪. لذلك ، استنتج أن تركيبات زيت الزوديا الثلاثة فعالة كطارد ضد بعوض الزاعجة المصرية.

الكلمات الدالة: *Aedes aegypti* L.، حمى الضنك النزفية، Evodia suaveolens Scheff، طارد، زيت Zodia.

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Implementing OSCE Exam for Undergraduate Pharmacy Students: A Two Institutional Mixed-Method Study

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ABSTRACT

Introduction: This study evaluates undergraduate pharmacy students' and examiners' perceptions of implementing OSCE exam.

Methods: A sample of 185 undergraduate pharmacy students (138 from Zarqa University and 47 from Yarmouk University) and 20 examiners were invited to complete a quantitative survey and qualitative focus group discussion, respectively.

Results: 103 out of 185 (56%, response rate) undergraduate pharmacy students completed the quantitative survey, with 11 examiners out of 20 (55%) agreeing to participate in the examiners' focus group discussion. Most pharmacy students agreed that OSCE exam was a practical and useful experience (74.8%) and should be part of the assessment in other pharmacy courses (61.2%). However, less than a quarter thought that OSCE exam was not fair (17.5%), very intimidating (20.4%), and needed more time (29.1%). Examiners were generally in favour of OSCE exam being well-organised and well-administered despite the need for a large place to conduct and a good number of pharmacy staff to implement.

Conclusion: Pharmacy students and examiners agreed that OSCE exam is an excellent and preferable clinical assessment tool. This study provides a scheme to evaluate OSCE exam as a clinical assessment tool and would help policy-makers gain more insight into the impact of implementing OSCE exam on students' clinical knowledge and communicational skills development and learning process.

Keywords: Clinical Performance, Pharmacy Education, OSCE Exam, Undergraduate Pharmacy Students, Mixed-Method, Pharmacy Training.

1. INTRODUCTION

Advances in pharmacy practice have transformed pharmacists' role from traditional dispensing to more patient-centred care practices (1–3). Pharmacists are currently assigned to provide pharmaceutical care services focusing on patient interviewing, taking a medication history, identifying medication-related problems, and designing an evidence-based pharmaceutical care plan (2,4–6). The advancement of the pharmacist's role should be met by restructuring the pharmacy education and assessment from focusing only on medicine compounding, selling, and dispensing to include pharmaceutical care provision (7–11).

The undergraduate pharmacy education in Jordan has changed tremendously, evidenced by the shift from a customer-based approach to a patient-centered approach (2,12–14). However, pharmacy schools in Jordan still

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adopt the traditional approach for evaluating undergraduate students' clinical competence (14). This approach has many limitations, such as low standardisation, subjectivity in evaluation, time-consuming, and testing of a limited number of intended learning outcomes (7,15). Such limitations posed a challenge that necessitates the search for standardised, more effective, and objective performance-based assessment tools (16,17).

Objective Structured Clinical Examination (OSCE) exam is a valuable tool for evaluating medical, pharmacy, and nursing students' clinical skills performance, and many studies have focused on using and introducing OSCE earlier in the pharmacy curriculum (18–22). The significance of OSCE exam is that it measures cognitive learning, essential practice skills, and the ability to communicate effectively using problem-solving skills (23–25). Pharmacy Schools at Zarqa and Yarmouk University have adopted OSCE exam as part of the curriculum change toward enhancing students' capabilities to provide a patient-centered approach to evaluate undergraduate pharmacy students' clinical and communicational skills and based on the recommendations of the pharmacy training committee and considering students' and academic staff feedback. Other universities, such as Jordan University of Science and Technology and the University of Jordan, have adopted OSCE exams for evaluating undergraduate Pharm D students. For example, data from the study conducted to assess the first-time implementation of OSCE exam for undergraduate Pharm D students at the University of Jordan showed a positive influence of OSCE exam on both students and pharmacy staff and opened the door for the implementation of OSCE at pharmacy schools in Jordan (26). This study aimed to evaluate quantitatively the undergraduate pharmacy students and qualitatively the examiners' perceptions toward the OSCE exam implementation in pharmacy schools of two Jordanian universities using a mixed method design.

2. METHODS

2.1 Compliance with ethical standards: This study was approved by the University Ethics Committee (supplementary material), and was given an approval number (1/3/2019-2020). Also, an informed consent form was obtained from all participants, ensuring that participation was voluntary and participants could withdraw at any stage, with their answers treated confidentially.

2.2 Study design setting and OSCE exam implementation

This is a mixed-method study where a quantitative survey (from Zarqa and Yarmouk universities) and qualitative focus group discussion (only from Zarqa University) were used to collect perceptions from undergraduate pharmacy students and examiners about OSCE exam implementation directly after finishing OSCE exam.

The undergraduate pharmacy students who underwent OSCE exam as a part of a compulsory exam of a 3-credit pharmacy training course at Zarqa University and a 3-credit pharmaceutical care course at Yarmouk University were invited to participate. Data were collected from 103 undergraduate pharmacy students who agreed to participate. OSCE exam, which was conducted at the end of the summer (August 2021) semester (Zarqa University) and the end of the second (May 2021) semester (Yarmouk University) of the academic year (2020-2021), was composed of two stations (i.e., mini OSCE). The first station consists of a multiple-choice question focusing on the required knowledge that pharmacy students should have, such as generic and brand names, primary care diseases, and rational medication use. The second station focused on simulated patient case scenarios, and pharmacy students were required to provide patients with oral, face-to-face pharmaceutical care. The clinical pharmacy training committees (i.e., at Zarqa and Yarmouk universities) that designed OSCE exam had two academics (i.e., HA and MN) who already had experience in OSCE

implementation. Also, the OSCE exam setup was reviewed by two Academics, one physician, and one community pharmacist.

An anonymous survey to assess the student's perception toward OSCE exam implementation was delivered by hand along with pharmacy students' feedback on the same day (i.e., after the OSCE exam was finished) and was filled out by undergraduate pharmacy students from the two universities. The survey was developed based on a comprehensive literature review (21,27–33) and modified according to the experts' (three senior academics, one community pharmacist, and one physician)) feedback to fit the study aims. The experts commented on the survey items' wording, clarity, and comprehensiveness and whether each item was relevant to the study aims. The authors reviewed the experts' feedback and comments and used them to refine the final version of the survey. The first section of the survey asks about student background information (i.e., educational level, age, and gender). The second, third, fourth, and fifth sections of the 3- point Likert scale survey (i.e., agree, neutral, and disagree) ask about OSCE exam attributes, performance, objectivity, scores, and outcomes.

After finishing the OSCE exam, examiners were invited to a group discussion to explore their perception of the OSCE exam implementation. The focus group discussion interview guide was developed based on the determined OSCE exam setup (i.e., mini OSCE) and the aims of this study and reviewed by two academic experts on qualitative research. During the "group discussion," examiners were asked general questions about exam implementation, logistics, and outcomes (supplementary material).

2.3 Data analysis

Descriptive statistics were used to analyse the undergraduate pharmacy student's survey results using SPSS (V23). Students' scores and regression analysis were also conducted. The focus group discussion (only at Zarqa University) was audio-recorded, and transcription was produced verbatim by two researchers (HA and MN). The transcription data were read and re-read before being qualitatively analysed and coded manually by HA (i.e., the moderator/facilitator of the focus group discussion) using thematic analysis into major and minor themes according to the six phases described by Braun and Clarke (34) and reviewed by MN. The results (i.e., the major and minor themes) were shared with our 11 examiners as part of the checking and validation process to ensure the data's accuracy, resonance, and credibility.

3. RESULTS

3.1 Student Perceptions (Quantitative part)

3.1.1 Demographics data

A sample of 185 pharmacy students (138 from Zarqa University and 47 from Yarmouk University) was targeted, with only 103 (73 from Zarqa University and 30 from Yarmouk University) pharmacy students completing the survey (response rate 56%).

The pharmacy students' ages range from 19 to 45 years. Around two-thirds (N=72, 70.6%) studied pharmacy at Zarqa University, and the vast majority of students were female (N=79, 76.7%), and around half were in their fifth (last) year of study (N=53, 51.5%). Only one-third had a pharmacy diploma before completing a bachelor's degree. Demographic data were summarised in Table (1). Six students reported being in their first, second, and third years held a pharmacy diploma before enrolling in the third-year level and were all studying at Zarqa University.

Table 1: Characteristics of the study sample (N=103)

Parameter	N (%)
***Age in years**	
18 – 26	89 (86.4%)
27 – 40	8 (7.8%)
> 40	2 (1.9%)
***Gender**	
Male	19 (18.4%)
Female	79 (76.7%)
***University**	
Zarqa Private University	72 (70.6%)
Yarmouk Governmental University	30 (29.4%)
Having a pharmacy Diploma before studying for a Bachelor of Pharmacy degree	
Yes	29 (28.2%)
No	74 (71.8%)
***Bachelor of pharmacy year level of study**	
First-year (holding diploma in pharmacy)	2 (1.9%)
Second-year (holding diploma in pharmacy)	1 (1.0%)
Third-year (holding diploma in pharmacy)	3 (2.9%)
Fourth-year	39 (37.9%)
Fifth-year	53 (51.5%)

* Some data was missing; subsequently, totals do not always add to 103.

3.1.2 Pharmacy Students' Perceptions of OSCE exam attributes, performance, scoring, objectivity, feedback, and outcomes

As shown in Table 2. The majority of pharmacy students agreed on OSCE exam attributes; they were given full OSCE exam directions (N=61, 59.2%), OSCE exam was well administered (N=67, 65.0%), and OSCE exam allowed them to highlight their areas of weaknesses (N=64, 62.1%). On the other hand, a few pharmacy students thought that OSCE exam was not fair (N=18, 17.5%), very intimidating (N=21, 20.4%), and needed more time (N=30, 29.1%). Regarding OSCE exam performance, the majority of pharmacy students agreed that instructions given before and during starting OSCE exam were clear (N=71, 68.9%), beneficial (N=72, 69.9%), and provided the opportunity to learn (N=70, 68%). Also, most of the pharmacy students agreed that OSCE exam was practical and gave them useful experience (N=77, 74.8%), communication skills were essential during OSCE exam (N=87, 84.5%), and feedback given after OSCE exam was necessary for learning (N=78, 75.7%). Finally, when asking about OSCE exam outcomes, most of the students agreed that OSCE exam made them aware of the types of mistakes that could happen during the dispensing process in real practice (N=74, 71.8%), counselling process (N=72, 69.9%) and that OSCE exam should be included as an assessment method in other pharmacy classes (N=63, 61.2%).

Table 2: Pharmacy Students' perceptions of OSCE exam attributes, performance, scoring, objectivity, feedback, and Outcomes (N=103)

Questionnaire Item	Response		
Points of evaluation	**Disagree, N (%)**	**Uncertain, N (%)**	**Agree, N (%)**
The OSCE attributes			
Full directions were given about OSCE by the school of pharmacy staff	5 (4.9%)	37 (35.9%)	61 (59.2%)
The OSCE was well administered by the school of pharmacy staff	7 (6.8%)	28 (27.2%)	67 (65.0%)
The OSCE is well structured and sequenced by the school of pharmacy staff	7 (6.8%)	35 (34.0%)	61 (59.2%)
The OSCE was appealing	3 (2.9%)	38 (36.9%)	60 (58.3%)
The OSCE was less stressful than other exams	46 (44.7%)	23 (22.3%)	33 (32.0%)
The OSCE allowed you to highlight the area of weaknesses you have as a pharmacy student	8 (7.8%)	31 (30.1%)	64 (62.1%)
The OSCE performance			
Instructions were given clearly *before* the OSCE	10 (9.7%)	22 (21.4%)	71 (68.9%)
Instructions *before* the OSCE was beneficial for the exam performance	5 (4.9%)	26 (25.2%)	72 (69.9%)
Instructions *during* the OSCE were clear and unambiguous	11 (10.7%)	31 (30.1%)	61 (59.2%)
The OSCE provided you with opportunities to learn	7 (6.8%)	22 (21.4%)	70 (68.0%)
The OSCE scoring, objectivity, and feedback			
The OSCE was practical and useful experience	5 (4.9%)	21 (20.4%)	77 (74.8%)
Communication skills are essential during the OSCE	5 (4.9%)	11 (10.7%)	87 (84.5%)
OSCE scores were affected by personalities and social relations	15 (14.6%)	20 (19.4%)	68 (66.0%)
Feedback given after OSCE by the pharmacy staff was clear and unambiguous	11 (10.7%)	31 (30.1%)	61 (59.2%)
Feedback given after OSCE was necessary for learning	6 (5.8%)	17 (16.5%)	78 (75.7%)
Feedback given after OSCE was objective and not subjective	7 (6.8%)	33 (32.0%)	60 (58.3%)
Outcomes of the OSCE			
The OSCE made students aware of the types of mistakes that can be made in the course of pharmacists' dispensing process	4 (3.9%)	22 (21.4%)	74 (71.8%)
The OSCE made students aware of the types of mistakes that can be made in the course of a pharmacists counseling process	3 (2.9%)	25 (24.3%)	72 (69.9%)
The OSCE should be included as an assessment method in other pharmacy classes	8 (7.8%)	29 (28.2%)	63 (61.2%)

3.1.3 Assessment of factors affecting pharmacy students' perception toward implementing OSCE exam as a clinical performance-based assessment tool

As shown in Table 3, pharmacy students at Zarqa University were more uncertain than pharmacy students at Yarmouk University in that; the pharmacy staff well-administered OSCE exam ($p = 0.025$), OSCE exam setting, and content at each station were compatible with real situations ($p = 0.009$), and OSCE exam allowed to compensate pharmacy students weaknesses area ($p =$

0.014). Also, pharmacy students at Yarmouk University were more agreed than pharmacy students at Zarqa students in that; OSCE exam covered broad areas of pharmacy training ($p = 0.04$), allowed them to highlight their areas of weaknesses ($p < 0.001$), instructions were given clearly before OSCE exam ($p = 0.02$), the time at each station was adequate ($p < 0.001$), and that OSCE exam made pharmacy students aware of the types of mistakes during dispensing and counselling processes ($p = 0.011$, 0.005 respectively). Lesser-year students were more uncertain than fifth-year students in that; OSCE exam setting and content at each station were compatible with real situations ($p = 0.004$) and awareness of the nature of OSCE exam ($p = 0.049$). Alternatively, fifth-year pharmacy students were more agreed than lesser years students in that; instructions were given clearly before OSCE exam ($p = 0.008$), instructions were beneficial ($p = 0.003$), and clear and unambiguous ($p < 0.001$).

Table 3: Assessment of factors affecting pharmacy students' perception toward implementing OSCE exam as a clinical performance-based assessment tool (N=103)

Parameter	Demographic variable (p-value, comments)				
	Gender	**Age groups**	**University**	**Studying year**	**Diploma before BSc**
The OSCE attributes					
Full directions were given about OSCE by the school of pharmacy staff	0.08	0.69	0.014 Zarqa students were more uncertain than Yarmouk students	0.02 Fifth-year students agreed more than lesser-year students	0.35
Awareness about the level of information needed for OSCE was appropriate	0.33	0.11	0.09	0.24	0.11
The OSCE was well administered by the school of pharmacy staff	0.01 Females agreed more than males	0.41	0.025 Zarqa students agreed more than Yarmouk students	0.19	0.12
The OSCE is well structured and sequenced by the school of pharmacy staff	0.12	0.48	0.76	0.85	0.23
The OSCE setting and content at each station were compatible with real situations (authentic)	0.92	0.64	0.009 Zarqa students were more uncertain than Yarmouk students	0.004 Lesser-year students were more uncertain than the fifth-year students	0.73
The OSCE was fair	0.21	0.29	0.112	0.31	0.36

Parameter	Demographic variable (p-value, comments)				
	Gender	**Age groups**	**University**	**Studying year**	**Diploma before BSc**
The OSCE was very intimidating	0.07	0.56	<0.001 Yarmouk students disagreed more than Zarqa students	0.12	0.52
The OSCE was very stressful	0.15	0.44	<0.001 Zarqa students agreed more than Yarmouk students	<0.001 Lesser-year students agreed more than the fifth-year students	0.16
The OSCE was appealing	0.56	0.99	0.26	0.37	0.14
The OSCE was less stressful than other exams	0.32	0.38	<0.001 Zarqa students disagreed more than Yarmouk students	0.03 Lesser-year students disagreed more than the fifth-year students	0.04 Students with Diplomas disagreed more than those not having diplomas before BSc.
More time was needed	0.35	0.34	0.029 Zarqa students agreed more than Yarmouk students	0.58	0.72
The OSCE covered broad areas of pharmacy training	0.49	0.65	0.04 Yarmouk students agreed more than Zarqa students	0.59	0.37
The OSCE allowed you to highlight the area of weaknesses you have as a pharmacy student	0.89	0.75	<0.001 Yarmouk students agreed more than Zarqa students	0.17	0.016 Students not having Diplomas agreed more than those with diplomas before BSc.
The OSCE allowed you to compensate for the area of weaknesses you have as a pharmacy student	0.10	0.51	0.014 Zarqa students were more uncertain than Yarmouk students	0.12	0.15

Parameter	Demographic variable (p-value, comments)				
	Gender	**Age groups**	**University**	**Studying year**	**Diploma before BSc**
The OSCE performance					
Awareness of the nature of the OSCE	0.61	0.50	0.008 Yarmouk students agreed more than Zarqa students	0.05 Lesser-year students were more uncertain than the fifth-year students	0.36
Instructions were given clearly *before* the OSCE	0.16	0.24	0.002 Yarmouk students were more agree than Zarqa students	0.008 The fifth-year students agreed more than lesser-year students	0.31
Instructions *before* the OSCE were beneficial for the exam performance	0.33	0.89	0.07	0.003 The fifth-year students year agreed more than lesser-year students	0.53
The OSCE tasks reflected those taught	0.91	0.29	0.002 Yarmouk students agreed more than Zarqa students	0.04 The fifth-year students agreed more than lesser-year students	0.33
Time at each station of the OSCE was adequate	0.08	0.17	<0.001 Yarmouk students agreed more than Zarqa students	0.13	0.48
Instructions *during* the OSCE were clear and unambiguous	0.36	0.22	<0.001 Yarmouk students agreed more than Zarqa students	<0.001 The fifth-year students agreed more than lesser-year students	0.12
Tasks asked during the OSCE were fair	0.96	0.74	0.68	0.80	0.34
The sequence of stations was logical and appropriate	0.07	0.78	0.06	0.25	0.26

Parameter	Demographic variable (p-value, comments)				
	Gender	**Age groups**	**University**	**Studying year**	**Diploma before BSc**
The OSCE provided you with opportunities to learn	0.70	0.84	0.003 Yarmouk students agreed more than Zarqa students	0.38	0.27
The OSCE scoring, objectivity, and feedback					
The guidance about the OSCE scoring system was given	0.21	0.98	0.75	0.68	0.47
The OSCE scores provide a true measure of essential clinical skills required for you as a pharmacy student	0.63	0.16	0.73	0.89	0.06
The OSCE scores were standardized	0.91	0.20	0.06	0.21	0.89
The OSCE was practical and useful experience	0.19	0.32	0.003 Yarmouk students agreed more than Zarqa students	0.26	0.11
Communication skills are essential during the OSCE	0.20	0.94	0.017 Yarmouk students agreed more than Zarqa students	0.78	0.38
OSCE scores were affected by personalities and social relations	0.24	0.89	0.003 Yarmouk students agreed more than Zarqa students	0.16	0.85
Feedback given after OSCE by the pharmacy staff was clear and unambiguous	0.77	0.50	<0.001 Yarmouk students agreed more than Zarqa students	0.13	0.07
Feedback given after OSCE was necessary for learning	0.19	0.87	0.024 Yarmouk students agreed more than Zarqa students	0.56	0.67

Parameter	Demographic variable (p-value, comments)				
	Gender	**Age groups**	**University**	**Studying year**	**Diploma before BSc**
Feedback given after OSCE was objective and not subjective	0.21	0.73	0.003 Yarmouk students agreed more than Zarqa students	0.60	0.78
The overall quality of the feedback given to you after the OSCE was good	0.19	0.56	0.21	0.48	0.52
Outcomes of the OSCE					
The OSCE made students aware of the types of mistakes that can be made in the course of the pharmacist dispensing process	0.81	0.78	0.011 Yarmouk students agreed more than Zarqa students	0.59	0.38
The OSCE made students aware of the types of mistakes that can be made in the course of a pharmacists counseling process	0.15	0.21	0.005 Yarmouk students agreed more than Zarqa students	0.95	0.91
The OSCE should be included as an assessment method in other pharmacy classes	0.36	0.27	0.001 Yarmouk students agreed more than Zarqa students	0.51	0.38

* Gender (reference; male), Age group (Reference; 18-26 years), University (Reference; Zarqa University), Studying year (Reference; first year), Having a pharmacy before (Reference; yes).

* Chi-Square correlation test χ^2 and Fisher exact tests were used to test the correlations.

The study shows positive students' OSCE perception scores of 33.13 (± 4.02) out of 44. Simple linear and multiple regression analyses were conducted to assess the correlation between demographic factors and the students' OSCE perception scores. Table 4 shows that all tested variables were not significant predictors for this score ($p > 0.05$).

Table 4: Assessment of different predictive factors affecting students' OSCE perception score (N=103)

Independent variables	Dependent variable: students' OSCE perception score			
	Standardised Coefficients Beta	**P-value#**	**Standardised Coefficients Beta**	**P-value$**
University type (Zarqa, Yarmouk)	0.023	0.068	0.170	0.161
Age group (18-26, 27-40, > 40)	0.010	0.731	0.062	0.551
Gender (male, female)	0.001	0.317	0.082	0.445

Independent variables	Dependent variable: students' OSCE perception score			
	Standardised Coefficients Beta	P-value#	Standardised Coefficients Beta	P-value$
Having a pharmacy Diploma before studying for a Bachelor of Pharmacy degree (Yes, No)	– 0.009	0.731	–0.080	0.485
Bachelor of pharmacy year level of study (1st, 2nd, 3rd,4th,5th)	0.027	0.829	0.080	0.506

#: using simple linear regression, $: using stepwise multiple linear regression.

3.2 Examiner's Perceptions (Qualitative part)

Four major themes were identified (table 5): "OSCE exam implementation and logistics," "OSCE exam objectivity and fairness," "OSCE exam compared to traditional examination methods," and "Advantages and disadvantages of implementing OSCE."

3.2.1 Examiner's Perceptions demographics data

The focus group discussion was completed with 11 examiners out of 20 (55%) who participated in the OSCE exam at Zarqa University. Examiners' age was between 26 to 45 years. Also, around (55%) of examiners were female. The academics comprised the majority of the examiners (64%), followed by community pharmacists (27%) and physicians (9%). Only three examiners (27%) had their first-time OSCE exam experience. Focus group discussion participants' characteristics were summarised in Table (4).

Table 4: Focus group discussion participants' characteristics at Zarqa University

Parameter	N (%)
Age in years	
26 - 35	3 (27%)
36 - 45	8 (73%)
Gender	
Male	5 (45%)
Female	6 (55%)
Occupation	
Academics	7 (64%)
Community pharmacist	3 (27%)
Physician	1 (9%)
Experiences as OSCE examiner	
First time as an OSCE examiner[1]	3 (27%)
Experienced OSCE examiner	8 (73%)

[1] The first time as an OSCE examiner was all academics.

3.2.2 OSCE exam implementation and logistics

Examiners were in favour that OSCE exam was well-organised and well-administered. Regarding OSCE exam attributes, examiners felt that pharmacy students were given clear instructions before entering OSCE exam station and adequate time to provide pharmaceutical care. Also, examiners ensured that they were oriented before starting OSCE exam, organisers and invigilators allowed an easy flow of pharmacy students for each exam centre with little or no distractions, and patients were well-trained and informed about each clinical case scenario. Lastly, regarding OSCE exam logistics, examiners believed that OSCE exam was vast

and challenging to organise based on having 10 OSCE exam centres that require a large place and a good number of pharmacy staff to conduct and that each exam centre requires two examiners and one patient (i.e., 30 academic/non-academic staff). However, having ten exam centres allowed examining many pharmacy students in a short time. The following quotes illustrate the former points:

"There is a significant and clear organizational effort to implement OSCE exam. Also, the exam invigilators and the security person facilitate the OSCE exam flow " (Examiner 1, Male, Academic).

"Students were familiar with the exam directions. Also, I felt as an examiner that the OSCE exam flow was smooth, and the students were oriented and familiar with the case scenarios. " (Examiner 3, Male, Community pharmacist).

" Despite the tremendous administrational effort, I think having many exam centers is very challenging and requires a large number of examiners and patients. (Examiner 4, Female, Academic). "Yes, I totally agree; this makes the OSCE exam implementation more stressful and difficult" (Examiner 6, Male, Physician).

3.2.3 OSCE exam objectivity and fairness

Examiners were in favour that OSCE exam centres' environment reflected fair and well-developed real clinical case scenarios covering common diseases and clinical skills, and different competencies such as communicational skills, counseling and patient education, dose optimisation, adverse drug reactions, patient up or down referral, and responsible primary care management. The following quotes illustrate the former points:

"I think the clinical case scenarios were more practical and reflected the reality, and the students have shown good skills and abilities to respond to these cases" (Examiner 5, Female, Academic).

"The students showed good knowledge and communicational skills to deal with clinical case scenarios during OSCE exam" (Examiner 11, Female, Academic)

"The clinical case scenarios contain common diseases and medications related to primary care" (Examiner 8, Male, Community pharmacist). "I agree; I felt that students understand their role when referring patients back to the primary care physician" (Examiner 6, Male, Physician).

3.2.4 OSCE exam compared to traditional examination methods

Examiners favored the OSCE exam as preferable but more challenging to conduct compared to other clinical examination forms. Also, examiners believed that OSCE exam was more stressful for pharmacy students, who were sometimes intimidated by the exam. Despite this, examiners supported the idea that OSCE exam would uncover more strengths and weaknesses the pharmacy students might have compared to other forms of clinical examination and that the feedback provided to pharmacy students was essential in enhancing their self-development of clinical knowledge and skills. The following quotes illustrate the former points:

"Despite the difficulty of conducting OSCE exam and the fear and tension of some students, OSCE exam is far better than the traditional exams" (Examiner 10, Female, Academic).

"OSCE exam is significant considering the fact that pharmacist role is now more patient centred" (Examiner 7, Female, Academic).

"According to my experience as a community pharmacist, I do believe that pharmacy students should have more OSCE exams than the classical way of examinations" (Examiner 2, Female, Community pharmacist).

3.2.5 Advantages and disadvantages of implementing OSCE

Examiners were in favour that OSCE exam would positively impact pharmacy students' learning process in terms of clinical knowledge and communicational skills. However, OSCE exam would require pharmacy students to have good pharmacist-patient communication skills and clinical knowledge to perform well during OSCE exam. The following quotes illustrate the former points:

" OSCE exam measures both student's knowledge and

clinical skills. To be honest, I am thinking of implementing OSCE exam at our faculty of pharmacy" (Examiner 9, Male, Academic).

"Despite the logistical costs, time, and staff requirements, undoubtedly, OSCE advantages outweigh the disadvantages" (Examiner 2, female, Community pharmacist).

Table 5: Focus group discussion major and minor themes

1. OSCE exam implementation and logistics
OSCE exam was well-organised and well-administered
- Clear OSCE exam instructions.
- OSCE case scenario training
- OSCE exam orientation.
- OSCE exam implementation process.
- Adequate time per OSCE station.
- Easy OSCE exam flow.
OSCE exam required large numbers of staff and large space to conduct
- Ten OSCE exam centres.
- Examiners per OSCE station.
- Patient per OSCE station.
- Invigilators and pharmacy staff.
- A large number of pharmacy students.
Objectivity and fairness
OSCE exam clinical case scenarios covered common clinical and communicational skills
- OSCE exam centres environment.
- Primary care problems mimic real case scenarios.
- Clinical case scenarios competencies.
- Clinical case scenarios were well-developed.
- Clinical case scenarios were fair.
- Pharmacy students' feedback.
- Pharmacy students' self-development of clinical knowledge and skills.
OSCE exam compared to traditional examination methods
- OSCE exam is preferable.
- OSCE exam is more challenging.
- OSCE exam is more stressful.
- OSCE exam is more competency-based.
Advantages and disadvantages of implementing OSCE
- OSCE exam has a positive impact on the learning process.
- OSCE exam uncovered clinical knowledge.
- OSCE exam uncovered communicational skills.
- OSCE exam student performance.
- OSCE exam is stressful.
- OSCE exam is sometimes intimidating.

4. DISCUSSION

The impact of OSCE exam in evaluating student knowledge, communication, and clinical skills competencies was evident in many studies (35–38). OSCE exam has been used to assess students' knowledge gained (i.e., to "show how") (39–42), their ability to memorise and reproduce the information (i.e., students "know" and "know-how") (39–42), and making students more self-aware and encourages them to identify their strengths and weaknesses.

Findings from the quantitative part showed that most pharmacy students agreed on receiving full clear, beneficial OSCE exam directions; OSCE exam was practical and well administered, OSCE exam allowed them to learn, highlighted their areas of weaknesses, and identified the mistakes that occurred during dispensing and counselling process. However, less than a quarter of pharmacy students thought that OSCE exam was not fair, very intimidating, and required more time. This is consistent with the results of other studies (21,26,33,43,44). For example, results from a study evaluating the use of a community pharmacy-based OSCE to consider self-care clinical skills in first-year pharmacy students in the school of pharmacy University of Arizona (USA) showed that the majority of students believed that OSCE exam was fair, covers a wide clinical wide range of vital clinical skills and that OSCE exam provided a practical experience and valuable learning opportunity (43). Also, results from another study showed that most students believed that OSCE exam was fair, covered the necessary knowledge and competencies, and that OSCE exam was well-administered and well-organised (21). However, the majority of students in this study (21) believed that OSCE exam was stressful and intimidating, contrasting the results of our study. Lastly, results from a Jordanian study (26) that evaluated OSCE exam in undergraduate Pharm D students at Jordan University revealed that a significant number of students believed that the time for each station was inappropriate, which contrasts with the results from our study. Although the Jordanian study raised a concern that the time may be considered a problem affecting student concentration in the exam, one study (42) reported that increasing the station time had no significant impact on student's performance during OSCE exam.

Pharmacy students at Zarqa University were more uncertain than pharmacy students at Yarmouk University in that; OSCE exam was well-administered, the content at each station was compatible with real situations, and OSCE allowed to compensate for the area of pharmacy students' weaknesses. Also, pharmacy students at Yarmouk University agreed more than pharmacy students at Zarqa students in that; OSCE exam covered broad pharmacy training topics, instructions were given clearly, time was adequate, and OSCE exam made students aware of mistakes that occurred during dispensing and counselling processes. The reasons behind these differences were not evaluated in this study, which may be a limitation to this study; however, it may be related to the differences in the number of pharmacy students and examiners required and the type of pharmacy course (i.e., pharmacy training vs. pharmaceutical care).

Findings from the qualitative part showed that examiners were in favour that OSCE exam would positively impact pharmacy students' learning process in terms of clinical knowledge and communicational skills in which pharmacy students are required to perform well during OSCE exam. Also, examiners agreed that OSCE exam was preferable but more challenging to implement and that OSCE exam was more stressful for pharmacy students who were sometimes intimidated by the exam.

This study uses a mixed-method design to highlight pharmacy students' and examiners' perceptions about implementing OSCE exams for undergraduate pharmacy students. This study involved a good number of pharmacy students from two universities (one private and one governmental) in different areas in Jordan; however, the findings drawn from this study may not be generalizable. Pharmacy students were asked to complete the survey, and the examiners to participate in the focus group directly after the end of OSCE exam; this could be a limitation of this study.

Also, examiners examined only one station and were unaware of what was happening in other stations; this would affect their perception of various OSCE exam aspects and would be another limitation of this study. Lastly, having 11 examiners are slightly higher than what you expect in the focus group (generally 6-10 participants, and the cut line below 12) (45), and there is a possibility that examiners' would lead each other during focus groups despite the presence of a well-trained focus group moderator/facilitator and assistant. This would be another limitation of this study.

This study provides a scheme to examine OSCE exam as a clinical assessment tool for undergraduate pharmacy students and would help policy-makers gain more insight into the impact of implementing OSCE exam on the development and learning process of students' clinical knowledge and communicational skills.

5. CONCLUSIONS

This study provided insight into implementing OSCE exam for undergraduate pharmacy students. Findings showed a positive perception of OSCE exam, which was perceived as a practical clinical assessment tool, and the implementation of the OSCE exam at Zarqa and Yarmouk universities was valuable and worthy. This would enlighten policy-makers about the significance of implementing OSCE exam for students' clinical knowledge and communicational skills development and learning processes. Future studies should focus more on clinical competencies and the OSCE exam's influence on pharmaceutical care and policy change.

Declarations

Author's contribution: Hamza Alhamad contributed to the design and conception of the study, acquisition of data, analysis and interpretation of data, drafting of the article, critically revising, and final approval of the version to be published. Deema Jaber and Mohammad B. Nusair contributed to analyzing and interpreting data, drafting the article, critically revising, and final approval of the version to be published. Finally, Fares Albahar, Sahar M Edaily, Nazek Qasim Al-Hamad, and Haneen Basheer contributed to data acquisition, analysis, interpretation of data, drafting of the article, and final approval of the version to be published.

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دراسة اثر تطبيق امتحان الاوسكي السريري على طلبة بكالوريوس الصيدلة باستخدام طرق بحثية مختلطة

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ملخص

المقدمة: تقيم هذه الدراسة تصورات طلاب الصيدلة الجامعيين والممتحنين حول تطبيق امتحان الاوسكي السريري.

منهجية البحث: تم استخدام تصميم الدراسة المختلطة ذات المنهج الكمي والنوعي في هذه الدراسة.

النتائج: شارك في هذه الدراسة 103 من اصل 185 طالب (نسبة المشاركة 56%) و11 من اصل 20 ممتحن (نسبة المشاركة 55%) اتفق معظم طلاب الصيدلة على أن تطبيق امتحان الاوسكي السريري كان تجربة عملية ومفيدة وأن اختبار الاوسكي يجب أن يكون جزءًا من التقييم في مواد الصيدلة الأخرى. ومع ذلك ، يعتقد نسبة قليلة أن اختبار الاوسكي لم يكن عادلاً ، ومخيفًا للغاية ، ويحتاج الطالب مزيدًا من الوقت اثناء الامتحان. اما بالنسبة لاراء الممتحنين فانهم يعتقدون أن امتحان الاوسكي السريري كان منظم جيدًا وتمت ادارة الامتحان على اكمل وجه على الرغم من الحاجة إلى مكان كبير لإجراء الامتحان وعدد كبير من موظفي الصيدلة لتنفيذه.

الخلاصة: اتفق طلاب الصيدلة والممتحنون على أن اختبار الاوسكي هو أداة تقييم سريرية ممتازة وبديل مفضل كامتحان يقيس المعلومات العلمية والمهارات السريرية. توفر هذه الدراسة منهجا لالية تطبيق امتحان الاوسكي كأداة للتقييم السريري وطريقا يساعد صانعي السياسات والتشريعات المرتبطة بمهنة الصيدلة على اكتساب المزيد من التبصر من خلال معرفة اثرتطبيق امتحان الاوسكي السريري على المعرفة السريرية للطلاب وعلى تنمية مهارات الاتصال وعملية التعلم لديهم.

الكلمات الدالة: تقييم الأداء السريري، التعليم الصيدلي، امتحان الاوسكي، طلاب بكالوريوس صيدلة، المنهج المختلط، التدريب الصيدلي.

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Substance Abuse among University Students: Assessing Prevalence, Risk and Preventive Measures

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ABSTRACT

Objectives: This research was undertaken to determine the prevalence and beliefs about drug abuse among university students in Jordan and to recommend certain preventive measures for the problem of drug addiction.

Methods: A descriptive cross-sectional online survey was conducted in April 2021 and included 679 students from private and public universities in Jordan. Students were asked to fill out the study survey through social media (Facebook and WhatsApp).

Results: The study included a survey conducted among 679 students from private and public universities whereby two third of them were females and more than half were studying in medical and health departments. It was found that 7.1% of university students used drugs in their life including illicit drugs, alcohol and cigarettes. Also, the addictive students started using drugs at a mean age of 18 years old ± 3.9. Importantly, around half of the addictive students succeeded to quit using drugs, 20.8% reported not trying to quit, while 33.3% of them tried but could not quit. In addition, the findings of this study revealed that peer pressure (n= 657, 96.8%), and the lack of religious commitment (n= 654, 96.3%), were the most motivational factors for drug abuse. Finally, regression analysis showed that female gender (OR= 0.094, p-value <0.001), and studying in public university (OR= 0.496, p-value= 0.042) were considered protective factors against substance abuse.

Conclusion: Focusing on increasing the awareness of youths about the risks of using drugs is a major framework in the society. Our recommendations are to increase awareness among the students, parents and society about drug abuse.

Keywords: Students, substance use, knowledge, attitude.

INTRODUCTION

Substance use is generally known as a state produced by the continued and exceeding use of a certain substance (natural or synthetic), which causes periodic and chronic intoxication detrimental to the individual and to society [1]. Such substance has a range of psychological effects such as alterations in the person's mood, thought, perception, and behavior by influencing the central nervous system [2-4].

There is no denial that the rate of substance use among university students is increasing [5]. Students from developing countries are at a greater risk of acquiring substance use disorders due to lack of the necessary identification, treatment and control programs of substance use disorders within the institutions of higher learning [6]. Most developing countries experience rapid economic, social, and cultural transitions creating

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favorable conditions for the increase in socially disruptive substance use [7,8].

Substance use is a critical area of research. This is due to the implications of developing substance dependence at an early age affecting negatively the future of young people [2]. There are ten categories of drugs that may lead to drug abuse including anxiolytics, caffeine, hallucinogens, sedatives, inhalants, stimulants, hypnotics, cannabis, alcohol, opioids and unknown substances [9]. A number of epidemiological studies had been conducted to determine the attitudes towards substance use among university students in different countries. Students in Islamic Azad University in Iran were knowledgeable about the factors of addiction, preventive methods, and harmful effects of substance use [3,31]. A small fraction of students at Tabriz University in Iran reported using drugs to feel more adult or tried to be more popular with peers [10]. Students in Turkey had negative attitudes towards heroin and cocaine and positive attitudes towards cannabis [11].

Although Jordan is known for its conservative Islamic values, substance use has become increasingly common [12,28,29]. It was reported that tobacco is considered a "gateway drug" that may lead to alcohol, hashish, and other substance use as well high-risk behaviors in the long term in Jordan (Abdel-Qader et al. 2021). However, not only is nicotine a highly addictive substance in and of itself, research is making clear the connection between smoking cigarettes and substance abuse, including the role of cigarettes as a gateway drug and a higher likelihood of relapse among smokers who achieve sobriety [3,30]. In 2003, Jordan adopted the Framework Convention on Substances Control. However, reports have indicated that substance use among Jordanian youths is on the rise, with hashish use increasing among the 16-25 age groups [13]. According to estimates from the United Nations Office on Drugs and Crime (UNODC), the largest amount of Captagon (the brand name of the psychostimulant fenethylline) was seized in Syria, Jordan and Kingdom of Saudi Arabia in 2011 [14].

A chemical analysis of 124 seized Captagon tablets in Jordan indicated the absence of fenethylline and the presence of many contaminants in an indication of the serious harmful effects from drug abuse [15]. Nearly 40% of all adults aged 25 years or over reported having smoked at least 100 cigarettes during their lifetime with smoking prevalence rate of 48.2% for men and 5.1% for women [5]. Very little is known about the knowledge, attitudes, and beliefs regarding substance use among Jordanian university students. Thus, there is increasing need to address substance use among Jordanian university students to forestall future problems with drug abuse.

METHODS

Study design and population

A descriptive cross-sectional online survey was conducted in April 2021 and included 679 students from private and public universities in Jordan.

Questionnaire development

The questionnaire was constructed upon reviewing relevant literature [1,2]. The draft questionnaire was reviewed by three research experts for face and content validity to assess its relevance, specificity, and comprehensiveness The questionnaire was developed in English,; the language of instruction in the Jordanian universities

The final version of the questionnaire was divided into several sections addressing different topics of interest. The aim of first section was to collect demographics data about participants' characteristics. The second section involved substance abuse behavior by the study participants. Notably and as we mentioned previously that nicotine a highly addictive substance which can led to substance use, for this reason, in this section the authors included smoking and alcohol drinking with illicit drug abuse in one question. The third part was planned to assess factors associated with substance abuse from students' perspectives using 3-point Likert scale that ranges from highly impact to no impact.

The aim of the fourth section was to assess factors that prevented non-addictive students from abusing substances using 5-point Likert scale with answers that range from strongly agree to strongly disagree.

Data collection

The study was conducted via an online survey that was uploaded on the Google Form platform. A sample of eligible participants was invited to participate in the study from the private and public universities in Jordan (n=679) through social media (Facebook and WhatsApp). The covering letter stressed anonymity, confidentiality and explained the objectives of this study. The participants did not receive any benefit or payment for filling out the questionnaire. The inclusion criterion was subjects who were studying at Jordanian universities.

Ethical approval

This study was approved by the Research Ethics Committee in Applied Science University (ASU), Amman, Jordan on 21st March 2021 (No: 2021-PHA-13). The consent to participate was implied by the act of completing and returning the electronic survey.

Statistical analysis

Data was analyzed using statistical package for social science (SPSS) version 22 (SPSS Inc., Chicago, IL, USA). The descriptive analysis was undertaken using mean and standard deviations (SD) for continuous variables and percentage for qualitative variables. Screening of the factors affecting the use of illicit drugs, alcohol, or cigarette smoking was carried out using univariate and multivariate logistic regression. Following univariate logistic regression analysis, any variables found to be significant on the single predictor level (p-value< 0.25) were entered into the multiple logistic regression analysis to explore the variables that were significantly and independently associated with the use of these substances. Variables were selected after checking their multicollinearity, where tolerance values were > 0.1 and variance inflation factor (VIF) values were <10. The values were checked to indicate the absence of multicollinearity between the independent variables in regression analysis (p-value <0.05 was considered to be statistically significant). Cronbach's alpha (α) was used to evaluate the internal consistency of the questionnaire, with values ≥ 0.7 considered acceptable [16].

RESULTS

The questionnaire's internal consistency and reliability was assessed by measuring Cronbach's α values of 0.880 and 0.786 for the risk factors and preventive factors, respectively. The values indicate acceptable internal consistency.

During the study period, 679 students from different Jordanian universities responded to the electronic questionnaire. Around two-third of the students were females (n= 449, 66.1%), and more than half of them were above 20 years old. Students were recruited from both private (n= 410, 60.4%), and public universities (n= 269, 39.6%), and more than half of them were from medical or health related colleges. Only 39.3% (n= 276) reported to have attended a previous workshop about substance abuse. Results are summarized in Table 1. The results show that the prevalence of any illicit drug abuse, alcohol and/or cigarette among the participated students throughout their lives was 7.1% (n= 48). The mean age when staring the use of these addictive substances was 18 years old ± 3.9. Students were asked about their attempts to quit these substances, where 20.8% (n= 10) reported not trying to quit at all, while 33.3% of them (n= 16) tried but could not quit. Around half of the students succeeded to quit these substances (n= 22, 45.8%). Results are presented in Table 2.

Table 1. Sociodemographic characteristics of the study participants (n= 679)

Parameter	n (%)
Age (years) • ≤ 20 years • >20 years	298 (43.9) 381 (56.1)
Gender • Male • Female	230 (33.9) 449 (66.1)
University • Private University • Public University	410 (60.4) 269 (39.6)
Field of study • Medical or health field • Other	368 (54.2) 311 (45.8)
Did you receive any workshop about drug abuse? • No • Yes	412 (60.7) 267 (39.3)

Table 2. Substance abuse behavior by the study participants (n= 679)

Parameter	Mean (SD)	n (%)
Have you ever used illicit drugs, alcohol, or cigarette smoking? No Yes		631 (92.9) 48 (7.1)
How old were you when you started using addictive substance?	18.0 (3.9)	
Have you ever quit or tried to quit using the addictive substance? *+ No, I did not try Yes, I tried but I could not quit Yes, I tried and succeeded		10 (20.8) 16 (33.3) 22 (45.8)

SD= Standard deviation, * percentage was calculated out of 48

Students were asked to determine their perception towards a number of factors that may be associated with the risk of substance abuse (**Table 3**). Peer pressure (n= 657, 96.8%), lack of religious commitment (n= 654, 96.3%), lack of parents' support (n= 652, 96.0%), and having addictive parents (n= 649, 95.6%) were the most common reported risk factors as perceived by students. In contrast, using these substances to help them in concentrating while studying (n= 415, 61.1%) was the least factor associated with the risk of substance abuse.

Table 3. Factors associated with substance abuse from students' perspectives (n= 679)

Factors	Moderate/high impact n (%)
Personal related factors	
Feeling of maturity	553 (81.4)
Imitating a famous person	607 (89.4)
Emotional relationship failure	600 (88.4)
To get away from trouble	553 (81.4)
Lack of religious commitment	654 (96.3)
Loss of a dear one (brother, father, mother, friend…)	614 (90.4)

Factors	Moderate/high impact n (%)
Free time	635 (93.5)
Family related factors	
Lack of parents' support	652 (96.0)
Bad relationship between parents and children	637 (93.8)
Having addicted parent(s)	649 (95.6)
Parent's separation (divorce)	608 (89.5)
Social related factors	
Impact of media, TV programs and series	628 (92.5)
Peer pressure	657 (96.8)
Ease of access to drugs in area	593 (87.3)
Too much money available for the student	624 (91.9)
University related factors	
To help in concentrating while studying	415 (61.1)
Academic failure	606 (89.2)

TV: television

Non-addictive students were asked about the factors that prevented them from abusing substances (**Table 4**). Playing sports (n= 588, 93.2%), counseling and advice (n= 582, 92.2%), in addition to prayers and supplications (n= 567, 89.9%) were the most commonly reported preventive measure. For more details, refer to **Table 4**.

Table 4. Factors that prevented non-addictive students from abusing substances (n= 631)

Factors	Strongly agreed/agreed n (%)
Counselling and advice	582 (92.2)
Prayers and supplications	567 (89.9)
Increasing the number of educational programs and activities	561 (88.9)
Abandoning relationship with people who use illicit drugs	550 (87.2)
Playing sports	588 (93.2)
Listening to music	456 (72.3)
Engage in artistic activities	564 (89.4)
Not carrying a lot of money	469 (74.3)
Specifying the places to visit	539 (85.4)

TV: television

Finally, logistic regression analysis led to the identification of two predictors of substance abuse behavior among the participating students (**Table 5**). As seen in **Table 5**, female gender (OR= 0.094, p-value <0.001), and studying in public university (OR= 0.496, p-value= 0.042) were considered protective factors against substance abuse.

Table 5. Assessment of predictors of substance abuse behavior among the study participants (n= 679)

Parameter	Are you addict? [0: No, 1: Yes]			
	OR	**p-value#**	**OR**	**p-value$**
Age (years) • ≤ 20 years • >20 years	Reference 3.191	0.001^	2.049	0.061
Gender • Male • Female	Reference 0.086	<0.001^	0.094	<0.001*
University • Private University • Public University	Reference 0.607	0.128^	0.496	0.042*
Field of study • Medical or health field • Other	Reference 1.199	0.545	----	----
Did you receive any workshop about drug abuse? • No • Yes	Reference 0.615	0.138^	1.969	0.506

Using simple logistic regression, $ using multiple logistic regression, ^ eligible for entry into multiple regression analysis, * significant at 0.05 significance level

DISCUSSION

There are religious, legal and cultural constrictions about the sale and consumption of drugs for non-medical purposes in the Middle East [17]. Nevertheless, there is an increase in the demand and use of these drugs in the Middle East region including Jordan [11]. Moreover, the rate of drug use among students has witnessed an increase over the past years [18-20]. Notably, the number of the annual published researches about drug abuses and disorders in the Arab countries is still low, a matter that led to underestimating the problem in several countries [20]. Unfortunately, youths are considered the most vulnerable group for substance abuse [21]. According to the National Strategy published by the National Council to Fight Drugs in the Hashemite Kingdom of Jordan (2009), the council recommended focusing on youths as they are considered the most vulnerable to drug addiction and represent the largest proportion of the Jordanian society [22]. Therefore, the aim of this study was to estimate the level, attitudes and beliefs about substance use among students in the Jordanian universities.

Our findings showed that in a sample of students from universities in Jordan (n=679), the percentage of drug abuse including illicit drugs, alcohol and\or cigarettes was 7.1%. A study reported that there was an association between cigarette smoking and substance abuse [23]. Another study revealed that cigarette is the way to alcohol and substance use [3]. Additionally, the findings of our study revealed that the mean age of start using these addictive drugs was 18 years old ± 3.9. In this context, Alzyoud and co-workers (2014) published that the rate of smoking cigarettes and waterpipe increased and involved 11-17 years old students in Jordan. According to their study, adolescents thought that it is safe to smoke tobacco then stop it two years later, a matter that led to the escalation of this problem [24]. However, this was not the main motivation for drug abuse in our study. The participation of 50% students from the medical and health field in the questionnaire conducted in this work can be a contributing factor to their understanding of drug adverse effects. Importantly, the respondents in this study mentioned that the main reasons for starting drug abuse included peer pressure, lack of religious commitment, lack of parents' support and having addictive parents. Other reasons

included imitating a famous person, feeling maturity, getting away of troubles, having free time and the impact of social media. Using drugs to increase the concentration of students was the least motivational factor for substance abuse in our sample of study. Earlier reports documented that there is positive correlation between depression in Jordanian university students and the use of painkillers, alcohol, caffeine, tobacco, tranquilizers, inhalants, and other substances [25]. Importantly, the results of this research are vital for recommending preventive measures for the problem of drug abuse between youths in Jordan. As it is clear from this study, the factors that led to drug abuse belong to several criteria including personal, social, family-related and university-related factors. Thus, we suggest introducing mandatory courses about drug addiction at the levels of schools and universities. The courses must cover drug addiction from many dimensions such as the religious, medical, social and cultural perspectives. Furthermore, arranging free lectures and awareness programs in the society can be an asset. In this context and due to the increase in the number of students who consume drugs in colleges and universities, it is importance to increase the awareness about drug abuse at school before students join colleges or universities [12]. Awareness programs can introduce many people to free-charge treatments from addiction in which many people are not knowledgeable about and have fear of approaching these treatment measures. Previous studies emphasized on the importance of targeting youths in drug awareness programs. Using attractive magazines and images that can refrain youths from approaching drugs can be an advantage. In this regard, Al Atom (2018) published cartoonish images for several pamphlets that target youths in Jordan and warn them from the side effects of using drugs such as "Drugs: your road to exhaustion." and "Drugs: Straight to Hell" [22]. Additionally, there are programs supported by the Jordanian Government to increase awareness about the risks of drug abuse such as the program of Drug-Prevention/Drug's negative effects on the Badia [22]. In addition, we recommend concentrating on strengthening the factors that prevented some students from approaching drug addiction in this study such as counseling/advice, prayers/supplications, educational programs/activities, playing sports, engaging in artistic activities, and specifying the places to visit. Haddad and colleagues (2010) conducted a survey among 400 high school students from the North of Jordan about the awareness of the risks of drug abuse and recommended collaboration between policy makers, health staff and religious people to prevent drug abuse in Jordan [26]. Moreover, it was reported that self-medication by several prescribed and non-prescribed drugs is very common in Jordan [27]. Thus, pharmacists need good training and confidence in providing information about drug side effects and not depending on reading the medicine information leaflet by patients [27]. Based on several reports that showed the increase in drug abuse in Jordan, Jaber et al. (2015) conducted a study about the opinion of Pharmacy students (at graduate and undergraduate levels) in Jordan regarding their training requirements to overcome the abuse of drugs including prescription drugs [28]. The students affirmed their needs for a structured training about identifying drug addicts, knowing drug types that are commonly abused in the area of the pharmacy as well as the way of dealing with drug addicts [28]. Accordingly, we recommend conducting such training so that the Jordanian pharmacists can collaborate with the authorities in fighting drug addiction.

Limitations of the study

Notably, there are some limitations in this study. First, the questionnaire was distributed randomly into the university students in Jordan in which 50% of the participant students were from the medical and health field and most probably are aware about the side effects of drug addiction. Different outcomes are expected if more respondents from other colleges answered the questionnaire. Second, the questionnaire was self-reported making it difficult to assess the truthfulness of the respondents' answers. Third, two third of the respondents were females and in a cultural restriction of a country in the Middle East, it is expected that addiction is less prevalent among females. Fourth, the mean age of the respondents was low, a matter that hinders generalizing the

data. Accepting these shortcomings, our study has considerable strengths as the data contribute to adding important information about drug addiction in Jordan where there is shortage in the studies conducted in this field. Also, the sample size obtained in this study is considered good. Most importantly, the study provides good recommendations for the authorities based on the responses of the participants.

CONCLUSION

Attention must be paid to the category of university students as there is increase in drug abuse in this class of the society. Our recommendations are to increase the number of educational programs and activities as well as to introduce mandatory courses about drug addiction in aim to raise the awareness of youths about the risks of using drugs.

Conflict of interests

The authors have no conflicts of interest to declare.

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تعاطي المواد المخدرة بين طلاب الجامعة: تقييم الانتشار والمخاطر والتدابير الوقائية

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ملخص

الأهداف: تم إجراء هذا البحث لتحديد مدى انتشار والمعتقدات حول تعاطي المخدرات بين طلاب الجامعات في الأردن والتوصية ببعض الإجراءات الوقائية لمشكلة الإدمان.

الطريقة: تم إجراء مسح وصفي مقطعي عبر الإنترنت في أبريل 2021 وشمل 679 طالبًا من جامعات خاصة وعامة في الأردن. طُلب من الطلاب ملء استبيان الدراسة عبر وسائل التواصل الاجتماعي (فيسبوك وواتس آب).

النتائج: اشتملت الدراسة على مسح تم إجراؤه على 679 طالباً من الجامعات الحكومية والخاصة، حيث كان ثلثاهم من الإناث وأكثر من نصفهم يدرسون في الأقسام الطبية والصحية. وجد أن 7.1٪ من طلاب الجامعات يتعاطون المخدرات في حياتهم من ضمنها المخدرات غير المشروعة والكحول والسجائر. أيضًا، بدأ الطلاب المدمنون في تعاطي المخدرات بعمر متوسط 18 عامًا ± 3.9. الأهم من ذلك، نجح حوالي نصف الطلاب المدمنين في الإقلاع عن تعاطي المخدرات، وأفاد 20.8٪ أنهم لم يحاولوا الإقلاع عن التدخين، بينما حاول 33.3٪ منهم الإقلاع عن التدخين، ولكنهم لم يتمكنوا من ذلك. بالإضافة إلى ذلك، كشفت نتائج هذه الدراسة أن ضغط الأقران (عدد= 657، 96.8٪)، وقلة الالتزام الديني (عدد= 654، 96.3٪)، كانت أكثر العوامل المحفزة لتعاطي المخدرات. أخيرًا، أظهرت تحليل النتائج أن جنس الإناث، والدراسة في الجامعات الحكومية تعتبر عوامل وقائية ضد تعاطي المخدرات.

الخلاصة: التركيز على زيادة وعي الشباب بمخاطر تعاطي المخدرات هو عمل مهم في المجتمع. توصياتنا هي زيادة الوعي بين الطلاب وأولياء الأمور والمجتمع حول تعاطي المخدرات.

الكلمات الدالة: الطلاب، تعاطي المخدرات، المعرفة، السلوك.

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Community Pharmacists' Attitudes, Preferences and Barriers toward Continuing Pharmaceutical Education: A Cross Sectional Study in Jordan

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ABSTRACT

Community pharmacists' responsibilities are expanding to foster optimal patient-centered care, which dictates postgraduate continuous education to enhance their competency and ability to face job challenges. We employed a cross-sectional online questionnaire-based study to evaluate community pharmacists' attitudes towards continuous education, their preferred modes of program delivery, factors they consider before joining a program, their preferred topics to learn about, and potential perceived barriers against continuous education.

A total of 358 community pharmacists completed the questionnaire. The majority of them (86.9 %) were interested in continuous education. However, most pharmacists (70.1%) had never attended any continuous education activity before. The most preferred type of delivery was self-learning through the internet (44.2%). Program cost and location were the major factors considered before accepting any activity (96.9%, and 96.6%, respectively). Among diseases, infectious disease was the most interesting topic for community pharmacists (92.7%). Regarding pharmaceutical topics, they were mostly interested in learning pharmacology and pharmacotherapy (94.1%), whereas pregnant and nursing mothers was the most desired patient group to learn about (92.2%). Job constraints and lack of time were the most reported barriers (89.4% and 89.1%, respectively).

Community pharmacists' have positive attitudes towards continuous education. However, many obstacles restrain them from effective participation in it. We provided sufficient data for policy makers to consider in future planning for continuous education activities that meet the needs of today's pharmacists to advance their practice.

Keywords: Community pharmacists, Continuous Education, Attitudes, Preferences, Barriers.

INTRODUCTION

The professional responsibilities of the community pharmacists have widely expanded in the recent years to be more patient focused via patient counselling, medication management, and preventive care practices[1]. Currently, pharmacists are recognized as professional health advisors and patients visit pharmacies for a more holistic approach to receive care [2-4]. Community pharmacists should keep competent all the way through their career in addition to maintain, update, and develop their knowledge, skills and capabilities to adeptly perform their job responsibilities and duties [5, 6].

Continuous education (CE), is described by Accreditation Council for Pharmacy Education (ACPE) as "a structured educational activity designed or intended to support the ongoing development of pharmacists and/or pharmacy technicians to maintain and enhance their

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competence" in delivering patient-centred care, working as part of interprofessional teams, practicing evidence-based medicine, focusing on quality improvement, using information technology, and developing and maintaining safe and effective medication use processes" [7]. These regulations are adopted in the United States in addition to several countries worldwide, where CE programs are mandatory and prerequisite to pharmacy license renewal [8-11].

Several studies have examined pharmacists' opinions and attitudes toward CE programs as well as barriers limiting pharmacists' involvement in these programs. A study conducted in the US reported that pharmacists found CE is an effective tool in improving their knowledge, and multiple types of CE programs were helpful resources to fulfil their educational needs [12]. Another study in Nepal showed that community pharmacists were interested in the CE program, and they felt that these programs help improving their knowledge about different aspects in pharmacy practice [13]. Another study found that pharmacists in Egypt were enthusiastic towards CE activity, however, community pharmacists attended less CE events relative to hospital pharmacists [14]. Pharmacists in Kuwait reported lack of time as the main barrier for attending CE programs [15].

In Jordan, some CE activities are infrequently held by the continuous education committee affiliated with Jordan Pharmacist Association. Leaders in the pharmacy profession were interviewed in one study to express their views on matters associated with education, practice, and pharmacy curricula status; where they stressed the importance of CE programs [16]. They also supposed that CE courses should be considered an obligation for license renewal. Moreover, pharmacy leaders suggested that CE programs are the most appropriate method to achieve pharmacist's competency in providing pharmaceutical care [16]. Moreover, an earlier study showed increased community pharmacists' demand for more education and training to handle patients' complaints in community pharmacy and have authorized prescribing role in Jordan [17]. Another study that included all pharmacy practice settings in Jordan including hospital, community, academic, and industry showed that 63.5% of the pharmacists were interested in CE activities, but they reported cost and poor timing as perceived barriers for their participation in CE programs [18].

While there is established evidence that CE is effective, CE programs are still suboptimal in Jordan and do not encourage intentional participation. Therefore, it is imperative to grasp the attitudes of community pharmacists towards CE, their preferences, and obstacles that impede them from joining CE activities, to allow for structured CE planning that better suit community pharmacist' needs. In this study we aimed to investigate community pharmacists' attitudes, preferences and barriers towards continuing pharmaceutical education programs and activities in order to guide future events related to effectively implement these programs and activities.

METHODS

Study Design and participants

The current cross-sectional study adapted a questionnaire from earlier studies of similar purposes (4, 16, 17). The questionnaire was sent online to 400 licensed pharmacy graduates who work in the community pharmacy setting across different geographical districts of Jordan in the period from August through December 2021. Raosoft® calculator showed that a sample size of 360 pharmacists was needed for a confidence level of 95% and a 5% margin of error [19]. The study received ethical approval from the Institutional Review Board at Jordan University of Science and Technology (JUST) (Reference number 20200240).

Study instrument

The first page of the survey included brief description of the study objectives and asked participants to confer their consent before filling the questionnaire. The survey

was divided into four parts; the first one included sociodemographic and practice characteristics including age, gender, marital status, highest degree obtained, university of graduation, number of years of practice, work schedule (full time vs. part time), geographical location of pharmacy, pharmacy type (single vs. chain), and role in pharmacy. The second part included questions to investigate pharmacists' preferences of CE programs. It started with asking pharmacists about their preferred method of conducting CE programs, their main criteria before joining a CE activity such as cost or topic of activity, and topics they would like to be included in CE such as diseases or specific patients' populations. Then, three closed questions assessed pharmacists' interest in CE programs, whether CE will improve their knowledge about medications, and their previous participation in CE programs. The third part investigated pharmacists' attitudes toward CE by requesting them to indicate if they agree or disagree with 5 statements about CE. The last part listed a variety of barriers which pharmacists may face to utilize CE programs. The survey was reviewed by the research team for face and content validity and modifications were made when appropriate. The questionnaire was also piloted on ten community pharmacists to ensure the clarity of the study questionnaire. The Cronbach's alpha coefficient was 0.664 indicating good internal consistency and reliability of the study instrument.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). Descriptive statistics were displayed by count and percentage for categorical variables and median ± interquartile range (IQR) for continuous variables. Univariate analyses were made using Chi-square test ($\chi 2$ test) or univariate binary logistic regression according to the number of factors for the independent variable. Multivariable binary logistic regression was used to find predictors of previous attendance of CE activities, adjusting for potential covariates.

RESULTS

A total of 358 community pharmacists completed the survey with a response rate of 89.5%. The median age of the participates was 29 years (IQR: 27-34). Most participants (n=295, 82.4%) were female, in the age group from 23 to 29 (n=217, 60.6%), had Bachelor of Pharmacy (n=210, 58.7%), and with less than 5 years of experience (n= 210, 58.7%). Table 1 lists socio-demographic and practice characteristics of the study participants.

Table 1: Socio-demographic and professional characteristics of participants (n=358).

Characteristic	Frequency (%)
Gender	
Female	295 (82.4)
Male	63 (17.6)
Age groups (years)	
23-29	217 (60.6)
30-39	121 (33.8)
40-49	20 (5.6)
Basic qualification in pharmacy	
Bachelor of Pharmacy	210 (58.7)
Master of Pharmacy	30 (8.3)
Doctor of Pharmacy (PharmD)	118 (33)

Characteristic	Frequency (%)
Years of work experience in the community pharmacy	
<5	210 (58.7)
5-9	72 (20.1)
10-14	49 (13.7)
15-19	19 (5.3)
>=20	8 (2.2)
Geographical area (Jordan)	
North	177 (49.4)
Middle	104 (29.1)
South	77 (21.5)
University of study	
Governmental-Jordan	283 (79)
Private-Jordan	68 (19)
Outside Jordan	7 (2)
Role in pharmacy	
Owner	31 (8.7)
Supervisor	167 (46.6)
Pharmacist	160 (44.7)
Type of pharmacy	
Single	185 (51.7)
Chain	173 (48.3)
Work schedule	
Part time	42 (11.7)
Full time	316 (88.3)
Marital status	
Married	190 (53.1)
Other	168 (46.9)

Pharmacists selected self-learning through the internet as their preferred modality to attend CE (n=158, 44.2%), followed by attending lectures and workshops (n=140, 39.2%). Scientific journals and graduate studies were chosen by only 8.7% and 7.9% (n=31 and n=29) of the pharmacists respectively. Figure 1 represents criteria considered by pharmacists when selecting CE activity to join. Community pharmacists acknowledged that they mainly consider CE program cost (n=347, 96.9%) and its location (n=346, 96.6%).

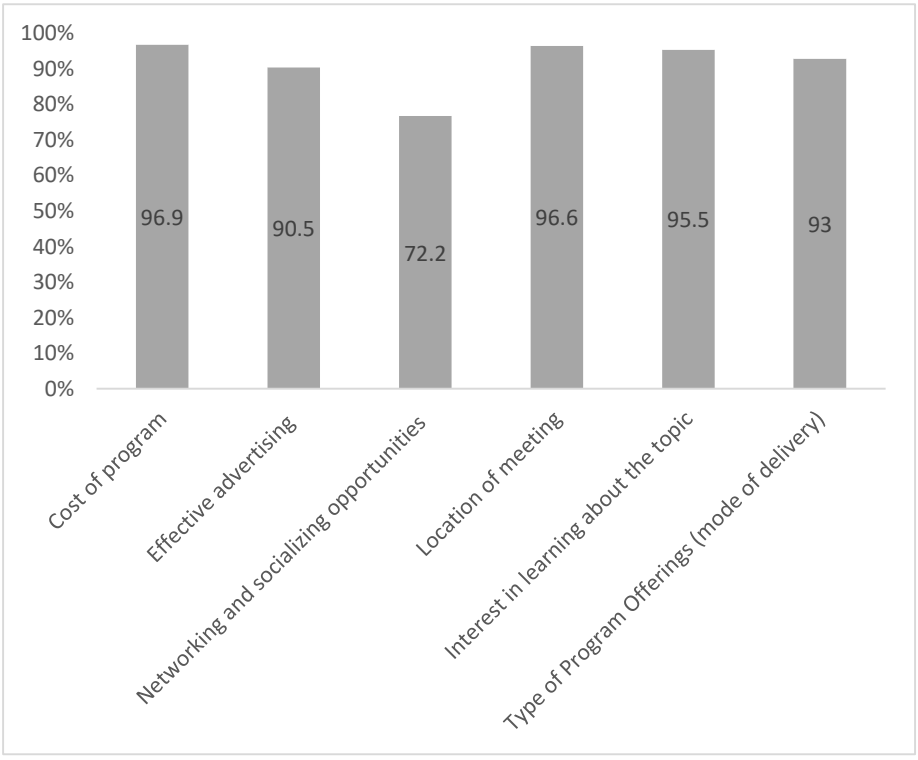

Figure 1: Factors affecting community pharmacists' choice of the CE program.

As presented in Figure 2, infectious diseases (n= 332, 92.7%), followed by gynaecologic disorders (n=321, 89.7%), were the major diseases of interest for the participating pharmacists, while hepatic and vascular diseases were the least cited topics (n=179, 50%). In terms of community pharmacy topics, community pharmacists opted for handling products as their major interest (n= 327, 91.4%), whereas pharmacy management was the least popular option of community pharmacists (n=307, 85.8%). Pharmacology and pharmacotherapy were ranked as the top pharmaceutical topic of interest for community pharmacists (n=337, 94.1%). On the other hand, pharmaceutical chemistry and microbiology were the least desirable topics (n=63, 17.7%). Regarding patients' population, most pharmacists were interested to work on pregnant and nursing mothers (n=330, 92.2%), followed by paediatric patients (n=328, 91.6%). Chronic disease patients were the least selected patients' group (n= 256, 71.5%).

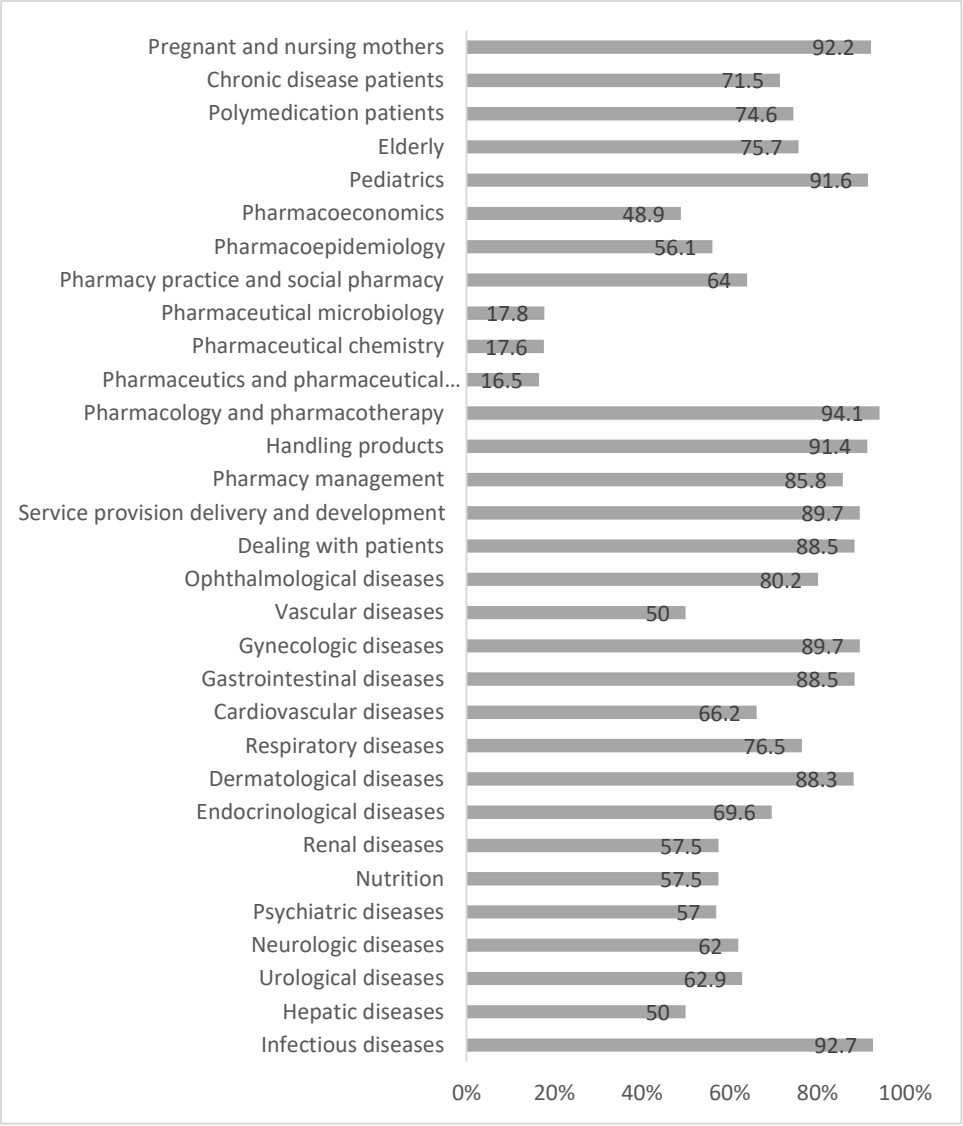

Figure 2: Preferred CE topics reported by community pharmacists.

Most of the participants (n=311, 86.9 %) reported their interest in attending CE activities and the majority of them (n=326, 91.1%) believed that CE programs are helpful in improving their knowledge about medications. However, only 29.9% (n=107) of the participants have previously attended CE programs. Most pharmacists agreed that CE improves their performance (n=314, 87.7%) and career prospects (n=276, 77.1%). Detailed community pharmacist's view about CE are shown in Table 2.

Table2: Community pharmacist's views about CE (n=358)

Statements	Frequency of the response (%)		
	Agree	Neutral	Disagree
Improves my performance in my current role	314 (87.7)	34 (9.5)	10 (2.8)
Enhances status of the profession with other health care professionals	312 (87.2)	42 (11.7)	4 (1.1)
Enhance my career prospects	276 (77.1)	43 (12)	39 (10.9)
Enhances status of the profession with the public	311 (86.9)	42 (11.7)	5 (1.4)
I see no benefits from CE	31 (8.7)	33 (9.2)	294 (82.1)

A shown in Table 3, univariate analysis showed that attendance of CE activities was significantly associated with older age (41-50 years), 5-9, 15-19 and >= 20 years of experience, holding master's degree, marital status, and being the owner of the pharmacy (P<0.05) (Table 3). After conducting multivariable analysis, holding master's degree (OR: 9.160, 95% CI: 3.656-22.950, p value= <0.0001), was the only significant predictor of CE attendance after adjustment for other variables (Table 3).

Table 3: Univariate and multivariable analysis of predictors of community pharmacists' previous CE attendance (n=358).

Characteristic	CE attendance, n (%)		Univariate analysis	Univariate analysis	Multivariable analysis	Multivariable analysis
	Yes	No	p value	OR (95% CI)	p value	OR (95% CI)
	107 (29.9)	251 (70.1)	-	-	-	-
Qualification						
Bachelor	57 (53.3)	153 (61)	Ref[c]	Ref	Ref	Ref
PharmD[a]	29 (27.1)	89 (35.5)	0.612	0.875 (0.521-1.468)	0.388	1.303 (0.715-2.376)
MSc[b]	21 (19.6)	9 (3.5)	0.0001*	6.263 (2.709-14.479)	0.000*	9.160 (3.656-22.950)
Years of experience						
<5	47 (44)	163 (65)	Ref	Ref	Ref	Ref
5-9	31 (29)	41 (16.3)	0.001*	2.622 (1.486-4.629)	0.125	2.001 (0.825-4.856)
10-14	12 (11.2)	37 (14.7)	0.751	1.125 (0.543-2.328)	0.719	0.776 (0.195-3.091)
15-19	10 (9.3)	9 (3.6)	0.006*	3.853 (1.480-10.036)	0.337	2.425 (0.397-14.818)
>=20	7 (6.5)	1 (0.4)	0.003*	24.277 (2.913-202.306)	0.222	10.574 (0.239-467.435)
Role in pharmacy						
Pharmacist	39 (36.4)	121 (48.2)	Ref	Ref	Ref	Ref
Supervisor pharmacist	54 (50.5)	113 (45)	0.112	1.483 (0.913-2.408)	0.444	1.252 (0.704-2.225)
Owner	14 (13.1)	17 (6.8)	0.021*	2.555 (1.155-5.653)	0.515	0.667 (0.198-2.253)
Marital status						
Other	35 (32.7)	133 (53)	Ref	Ref	Ref	Ref
Married	72 (67.3)	118 (47)	0.001*	2.319 (1.444-3.724)	0.212	1.453 (0.808-2.612)

Characteristic	CE attendance, n (%)		Univariate analysis	Univariate analysis	Multivariable analysis	Multivariable analysis
	Yes	No	p value	OR (95% CI)	p value	OR (95% CI)
Age						
23-30	59 (55.1)	176 (70.1)	Ref	Ref	Ref	Ref
31-35	22 (20.6)	43 (17.1)	0.162	1.526 (0.844-2.760)	0.627	1.289 (0.463-3.590)
36-40	17 (15.9)	30 (12)	0.121	1.690 (0.870-3.284)	0.300	2.131 (0.509-8.923)
>=41	9 (8.4)	2 (0.8)	0.001*	13.424 (2.820-63.899)	0.314	4.225 (0.218-81.979)

a: PharmD, Doctor of Pharmacy degree; b: MSc, master's degree; c: Ref, reference group

* Statistically significant differences (p value < 0.05).

Perceived barriers limiting pharmacists' participation in CE programs are shown in Table 4. Job constraints (n=320, 89.4%) and lack of time (n=319, 89.1%) were the most reported barriers, followed by the accessibility (location/distance) issues of group learning activities (n= 313, 87.5%). Only few of the participants (n= 36, 10.1%) stated that feeling of not belonging to the profession is the barrier for not seeking to attend CE activities.

Table 4: Perceived barriers that prevent community pharmacists from participating in CE activities (n=358).

Item	Frequency of the response (%)		
	Agree	Neutral	Disagree
Uninteresting subjects or topics	125 (34.9)	126 (35.2)	107 (29.9)
Lack of quality learning activities	294 (82.1)	50 (14)	14 (3.9)
Subjects/topics too specialized	49 (13.7)	175 (48.9)	134 (37.4)
Family constraints (e.g. spouse, children)	221 (61.7)	100 (28)	37 (10.3)
Lack of appropriate learning opportunities	294 (82.1)	50 (14)	14 (3.9)
CE is not a priority for me	45 (12.6)	32 (8.9)	281 (78.5)
Cost of participation	237 (66.2)	81 (22.6)	40 (11.2)
Lack of time	319 (89.1)	30 (8.4)	9 (2.5)
Job constraints	320 (89.4)	27 (7.5)	11 (3.1)
Accessibility (location/distance) of group learning activities	313 (87.5)	27 (7.5)	18 (5)
Feeling not belonging to the career	36 (10.1)	20 (5.6)	302 (84.3)

DISCUSSION

Community pharmacists are easily accessible members of the healthcare team who should keep competent to provide optimal health services for patients through CE. An earlier study evaluated the needs, barriers, and motivations of pharmacists to provide continuous education in Jordan [20]. However, the current study has exclusively evaluated community pharmacists' attitudes, preferences, and barriers to provide CE. Moreover, because pharmacists are an essential and most easily accessible part in healthcare systems, more than one study is needed to investigate pharmacists' preferences and barriers regarding CE.

Consistent with the current trends, community

pharmacists preferred the online self-learning activities in the present study. This is similar to a Lebanese study which found that the majority of pharmacists enrolled in CE system believed that online courses are easier to do and they have used them at least once [9]. Moreover, online CE programs were found to be the most frequently used among a sample of Texan pharmacists for their latest CE reporting period [8]. Another study in Kuwait found that seminar attendance was the most cited CE activity by pharmacists, whereas reading a journal article was least cited [15]. This may be attributed to lack of access to full journal article and difficulty of interpreting article results and applying them in practice. Community pharmacists' preference of online CE programs matches their reported barriers of job constraints and lack of time because they offer the flexibility compared with other CE programs modes; where they may be finished anywhere, with no travel requirements.

The cost of CE program and its location were the most important factors considered by participants before joining CE activity in this study. The limited salaries of community pharmacists as well as lack of employer's reimbursement for any cost they afford for joining CE programs could justify this finding. Interest in learning about the topic was also an important factor considered by the majority of the participants in the current study before attending a CE activity. Scope of programs was the first criterion considered by participants in a Texan study when selecting CE program, followed by location of the meetings. Most of the participants in the latter study also indicated that cost of CE programs is a significant criterion when selecting CE programs[8].

Results showed that community pharmacists prefer to learn more about pharmacotherapy,, infectious and gynaecologic diseases, and specific patients' groups such as pregnant and nursing mothers as well as paediatric patients'. This can be attributed to community pharmacists' growing job responsibilities which include their fundamental contribution to patient care. Accessibility of community pharmacists enable most consumers to visit a pharmacy for health advice which is available on demand[21]. Some countries indeed have legitimized community pharmacist prescribing role, which positively affected patient care and treatment outcomes[17]. A Lebanese study showed that pharmacists preferred treatment guidelines followed by medication therapy management as CE topics of interest[9]. Innovations in disease management was the most desirable topic of Malaysian community pharmacist[22].

Most community pharmacists in this study were interested in CE and believed it would improve their knowledge and practice. This finding is consistent with previous research in other countries [14, 15, 20, 23, 24], where participants have shown their enthusiasm about CE. However, only few of the surveyed community pharmacists have previously attended CE activity, particularly older pharmacists with higher qualification, which is similar to an earlier Kuwaiti study finding. [15].

Consistent with research findings in Egypt and Malaysia [14, 22], lack of community pharmacists' attendance for CE was primarily attributed to job constraints and lack of time; where most community pharmacists work 8 hours a day and 6 days per week. Lack of time was also a major barrier reported by community pharmacists in Kuwait[15]. In another English study, community pharmacists stated that working for late hours, far locations, and lack of reimbursement were the major obstacles limiting their participation in CE activities [25]. Likewise, work obligations were reported as a barrier by more than half of the Lebanese pharmacists [9]. Lack of appropriate learning opportunities in Lebanon was cited as a barrier by large number of participants. These findings reveal the urgent need to examine and adjust working circumstances of community pharmacists to allow for promoting and implementations of CE programs that fit them and boost their educational needs effectively. A previous study in Jordan found that community pharmacists are less satisfied with their jobs compared

with hospital pharmacist, which may be attributed to long working hours including weekends with no overtime reimbursement, discouraging work circumstances such as lack of advancement opportunities and poor relationship with physician[26]. Comparable reasons were responsible for job dissatisfaction among American community pharmacists as well[27], where they reported workload, management-related issues, and work/life balance as their top reasons. To overcome some of these challenges, an adequate number of pharmacists and pharmacists' assistants should be employed at each community pharmacy, with more flexible working hours. An earlier study suggested numerous interventions to improve pharmacy practice in the developing countries, including clear distinction between the roles and responsibilities of different pharmacists categories of (a graduate vs assistant pharmacist**)** and disallowing physicians from dispensing medicines[28]. Moreover, salaries of community pharmacists need to be appropriate to their workload.

Limitations in this study included the collection of responses through self-reported online questionnaires, thus participants may have responded in a way that is different from their actual attitudes. Also, the online survey has limited accessibility in some participants such as those who have limited internet access particularly in older age groups.

CONCLUSION

Although most of the community pharmacists were interested in CE, only few of them attended CE programs. Job restrictions and lack of time were the most common barriers to attend CE programs. Reducing working hours and allowing more flexible working time and distribution of job duties on a larger number of pharmacists and assistants in the community pharmacy should be considered by health policy makers to enhance pharmacists' participation in CE programs and hence their ability to provide the optimal health services in community pharmacy setting.

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مواقف صيادلة المجتمع وتفضيلاتهم ومعوقاتهم تجاه التعليم الصيدلاني المستمر: دراسة مقطعية في الأردن

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ملخص

تتوسع مسؤوليات صيادلة المجتمع لتشمل تعزيز الرعاية المثلى للمريض، مما يستوجب التعليم المستمر بعد التخرج لتعزيز كفاءتهم وقدرتهم على مواجهة تحديات العمل. في هذا البحث استخدمنا دراسة مقطعية عبر الإنترنت قائمة على الاستبيان لتقييم مواقف صيادلة المجتمع تجاه برامج التعليم المستمر، والطرق المفضلة لعقده، والعوامل التي يأخذونها في الاعتبار قبل الانضمام إليه، والموضوعات التي يرغبون بالتعلم عنها، والمعيقات المحتملة التي تحول دون التعليم المستمر . أكمل ما مجموعه 358 صيدلانيا من المجتمع المحلي الاستبيان وكان غالبيتهم يرغبون بالتعليم المستمر. ومع ذلك، فإن معظمهم لم يسبق لهم حضور أي نشاط تعليم مستمر من قبل. وأظهرت نتائج البحث أن أغلب المشاركين يفضلون التعلم الذاتي عبر الإنترنت وأن تكلفة البرنامج وموقعه من العوامل الرئيسية التي يأخذها صيادلة المجتمع في الاعتبار قبل المشاركة في أية أنشطة للتعليم المستمر. وكشف البحث أيضا أن الأمراض المعدية هي الموضوع الأكثر اهمية للصيادلة في المجتمع بالإضافة إلى علم الادوية والعلاج الدوائي وعلاج الأمهات الحوامل والمرضعات. أما عن المعيقات فإن قيود العمل وضيق الوقت هي أكثر العقبات التي تمنع المشاركة في التعليم المستمر حسب ما أشار إليه الصيادلة المشاركون بالبحث. أخيرا فإن صيادلة المجتمع لديهم مواقف إيجابية تجاه التعليم المستمر. ومع ذلك، فإن هناك عقبات كثيرة تمنعهم من المشاركة الفعالة فيها. لقد قدمنا في هذا البحث بيانات كافية لصانعي القرار للنظر فيها عند التخطيط المستقبلي لأنشطة التعليم المستمر التي تلبي احتياجات الصيادلة اليوم للنهوض بممارسة الصيدلة.

الكلمات الدالة: صيادلة المجتمع، التعليم المستمر، المواقف، التفضيلات، العوائق.

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Effect of COVID-19 on Liver Enzymes in Hospitalized COVID-19 Patients in the Gaza Strip: A Retrospective Study

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ABSTRACT

In severe cases, the novel coronavirus disease 2019 (COVID-19) can cause respiratory failure and multiple organ dysfunction, including liver injury. This study assessed the COVID-19 effect on liver function among hospitalized COVID-19 patients. Three-hundred and seventy patients were recruited. Patients were distributed as the following: control group (n=100), intensive care unit (ICU) hospitalized COVID-19 patients (n=140), and non-ICU hospitalized COVID-19 group (n=130). Data about the levels of liver enzymes were collected from the hospital medical records of the participants. Our results showed a significant increase in alanine aminotransferase (ALT) levels among the ICU hospitalized COVID-19 patients compared with the non-ICU hospitalized COVID-19 patients (p-value <0.01) and the controls (p-value <0.001). Aspartate aminotransferase (AST) concentration significantly increased among the ICU-hospitalized COVID-19 group compared with the non-ICU hospitalized COVID-19 group (p-value <0.01) and the controls (p-value <0.05). The ICU-hospitalized COVID-19 patients had a higher increase in alkaline phosphatase (ALP) levels compared to the non-ICU hospitalized COVID-19 patients and controls (p values <0.001). Based on ALT, AST, and ALP levels, we found that 73 (52%), 77 (55%), and 38 (27%) of the ICU hospitalized COVID-19 patients developed a liver injury. Of those, 12 (8.5%) died compared to 5 (3.5%) patients with abnormal liver function. In conclusion, these findings suggest that COVID-19 disease is associated with abnormal liver function and liver injury.

Keywords: COVID-19, SARS-CoV-2, Liver enzymes, Liver damage.

INTRODUCTION

A rapid outbreak of acute respiratory illnesses was observed in Wuhan, China, in December 2019 [1–6]. The China Novel Coronavirus Investigating and Research Team succeeded in isolating and identifying the causative agent of these respiratory diseases, which was called a novel coronavirus (2019-nCoV). The international committee on taxonomy of viruses later renamed it severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [4,7]. On March 7, 2020, the World Health Organization (WHO) announced a new name for the epidemic disease caused by SARS-CoV-2: Coronavirus disease (COVID-19), which has been proclaimed as a global pandemic [8]. On January 03, 2022, WHO reported that there have been 291,413,610 confirmed cases of COVID-19 globally, including 5,461,241 deaths [9]. According to Palestinian Ministry of Health statistics on January 03, 2022, there were 470656 confirmed COVID-19 cases (190538 in Gaza Strip) and 4947 confirmed deaths (1709 in Gaza Strip).

SARS-CoV-2 is a single-stranded RNA virus that binds to angiotensin-converting enzyme 2 (ACE2) strongly expressed by epithelial cells of the mouth, lower respiratory tissues, as well as to a lesser extent by the epithelial cells of other organs such as the heart, liver,

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kidneys and intestines [10]. SARS-CoV-2 infection has a wide range of symptoms, from asymptomatic to life-threatening conditions such acute respiratory distress syndrome and multiple organ failure. The majority of COVID-19 cases are mild, with the most common symptoms being fever, fatigue, and a dry cough [1,2,11–13]. Severe cases, on the other hand, can lead to organ dysfunctions such as lung injury, heart injury, liver injury, and kidney injury [1,2,11,12,14–16]. Patients with COVID-19 have aberrant liver function, according to recent studies, with raised alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) [2,12,14,17]. Interestingly, a histological evaluation of liver biopsy specimens from a COVID-19 patient revealed significant microvesicular steatosis as well as modest lobular and portal activity, showing that SARS-CoV-2 may trigger liver damage [18]. Additionally, COVID-19 individuals with pre-existing liver disease have a greater mortality rate than those who do not have pre-existing liver disease [19]. Regrettably, the actual mechanism underlying the development of COVID-19-related liver damage remains unknown. ACE2 receptors found in liver tissues may play a role in the progression of liver damage. Cholangiocytes exhibit a high expression of ACE2 receptors, according to a recent study, indicating that the SARS-CoV-2 virus may bind to ACE2 on cholangiocytes, inducing cholangiocyte malfunction and liver injury [20]. Liver injury is defined by an increase of over two times the upper limit of normal range in serum ALT or conjugated bilirubin, or a combined increase of AST, ALP, and total bilirubin, provided one of them is above the upper limit of the normal range. COVID-19 also causes severe acute systemic inflammatory responses and cytokine storms, that result in a permanent multi-organ damage[1,2,11,12,14]. Drug-induced liver injury is another possibility, as some COVID-19 patients are taking hepatotoxic medicines such as remdesivir, ritonavir, lopinavir, and chloroquine[14,18]. Recent information about the COVID-19 outbreak has begun to provide light on the impact of the COVID-19 disease on the liver [9,11,14]. However, few studies have analyzed variations in liver function tests among COVID-19 patients. This study aimed to evaluate the effect of SARS-CoV-2 infection on liver function among hospitalized COVID-19 patients.

MATERIAL AND METHODS

This study is a single-center cross-sectional retrospective study which assessed the effect of COVID-19 on liver function among COVID-19 patients who were admitted to the European Gaza Hospital in the Gaza Strip. This study was approved from the Helsinki Committee for Ethical Approval, Palestinian Health Research Council, Gaza, Palestine. The ethical approval number: PHRC/HC/1104/21.

Study population

Patients were included in the study if they were admitted with SARS-CoV-2 infection confirmed by the real-time polymerase chain reaction (RT-PCR) of nasal swab samples. In this study, 270 COVID-19 patients of both genders who were admitted to the European Gaza Hospital in the Gaza Strip, as well as 100 non-ICU patients with negative COVID-19 RT-PCR tests, free of liver diseases and any condition elevating liver enzymes levels have participated (Figure 1). The participants were divided into three groups: control group (100 people with negative COVID-19 PCR tests); non-ICU hospitalized COVID-19 patients (130 COVID-19 hospitalized patients were not admitted to the ICU); ICU hospitalized COVID-19 patients (140 COVID-19 hospitalized patients were admitted to the ICU). COVID-19 hospitalized patients and control group participants with pre-existing liver such as hepatitis, liver cirrhosis, or liver cancer were excluded from the study. Patients with missing clinical data or died before completing the biological tests for the liver function were excluded. In addition, individuals on regular hepatotoxic medications were also excluded from the study.

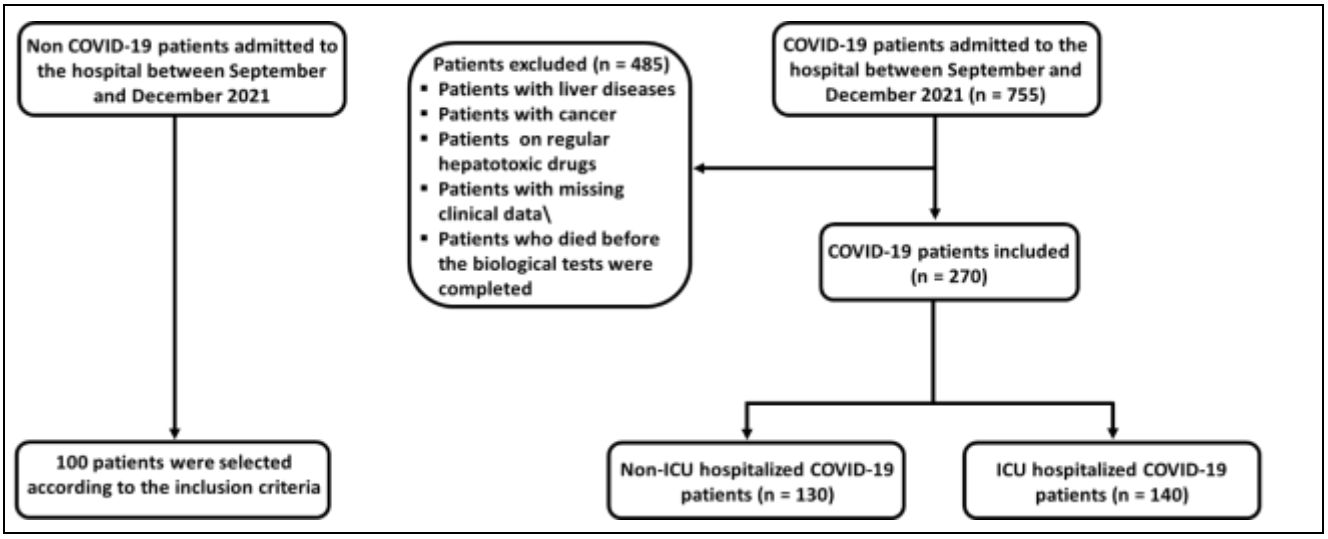

Figure 1: Patient flow chart.

Patient flow chart showing the total number of COVID-19 and non-COVID-19 patients admitted to the European Gaza Hospital in the Gaza Strip from September to December 2021. All participants were selected according to the inclusion criteria.

Data collection

During the study period, data was collected for each participant from the medical records at the European Gaza Hospital including patient demographics, and laboratory parameters of the liver function (ALT, AST and ALP). Clinical laboratory services at the European Gaza Hospital informed us that determination of serum levels of liver enzymes (ALT, AST and ALP) was performed using commercially available kits and according to manufacturer's instructions as the following: ALT kit (manufacturer: AMS – ITALY, Ref: GA492100, Lot: BB017CB); AST kit (manufacturer: AMS – ITALY, Ref: GA492100); ALP kit (manufacturer: Reactivos – SPAIN, Ref: EZ012LQ, Lot: LIQ-1148-M). Abnormal liver function tests were defined as: ALT >36 U/L, AST >35 U/L, ALP >120 U/L. Moreover, liver injury was defined in patients who had raised ALT, AST and/or ALP more than two times the upper limit unit of normal range[14].

Statistical analysis

Graphics and statistical analyses were performed using Graphpad Prism software (San Diego, CA, USA). Data was presented as mean ± SD. Comparisons between two different groups was performed using unpaired *t* test. For all tests, P values ≤ 0.05 were considered to be significant (* $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

Characteristics of the Study Population

This study comprised a total of 370 participants divided into three groups. The data in Table 1 showed no statistically significant differences in age and BMI between the study population. The levels of ALT, AST, and ALP in the non-ICU hospitalized COVID-19 patients and ICU hospitalized COVID-19 patients were significantly different from those in controls. In addition, COVID-19 patients admitted to the ICU showed a significant increase in the levels of ALT, AST, and ALP compared with COVID-19 patients who were not admitted to the ICU. In the ICU hospitalized COVID-19 patients' group, we also observed that 5 (3.5%) of COVID-19 patients with abnormal liver function tests and 12 (8.5%) of patients with liver injury died during their hospitalization as a result of COVID-19-induced respiratory failure or multiple organ dysfunction.

Table 1: Characteristics of the study population

Variable		Control group	non-ICU hospitalized COVID-19 patients	ICU hospitalized COVID-19 patients	P values
		n=100	n=130	n=140	
Age (year)	Mean ± SD (range)	60.83 ± 0.87 (52 – 70)	62.70 ± 1.1 (54 – 71)	64.93 ± 0.96 (56 – 72)	0.14*, 0.28†, 0.11‡
BMI (kg/m2)	Mean ± SD	24.12 ± 1.3	23.82 ± 1.4	25.03 ± 0.9	0.53*, 0.84†, 0.72‡
ALT (U/L)	Mean ± SD	21.77 ± 0.37	28.92 ± 0.88	44.82 ± 2.28	< 0.05*, < 0.01†, < 0.001‡
	Normal (n, %)	100 (100%)	91 (70%)	12 (9%)	
	Abnormal (n, %)	0 (0%)	39 (30%)	55 (39%)	
	Liver injury (n, %)	0 (0%)	0 (0%)	73 (52%)	
AST (U/L)	Mean ± SD	19.85 ± 0.35	30.72 ± 0.98	42.41 ± 1.90	< 0.05*, < 0.01†, < 0.001‡
	Normal (n, %)	100 (100%)	87 (67%)	10 (7%)	
	Abnormal (n, %)	0 (0%)	43 (33%)	53 (38%)	
	Liver injury (n, %)	0 (0%)	0 (0%)	77 (55%)	
ALP(U/L)	Mean ± SD	50.79 ± 1.13	84.78 ± 2.98	145.30 ± 3.90	< 0.01*, < 0.001†, < 0.001‡
	Normal (n, %)	100 (100%)	85 (65%)	14 (10%)	
	Abnormal (n, %)	0 (0%)	45 (35%)	88 (63%)	
	Liver injury (n, %)	0 (0%)	0 (0%)	38 (27%)	
Death	Normal liver function tests (n, %)	0 (0%)	0 (0%)	0 (0%)	
	Abnormal liver function tests (n, %)	0 (0%)	0 (0%)	5 (3.5%)	
	Liver injury (n, %)	0 (0%)	0 (0%)	12 (8.5%)	

ICU: Intensive care unit; BMI: Body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase. *) p-values which represent the comparison between the means of control group and the non-ICU hospitalized COVID-19 patients' group. †) p-values which represent the comparison between the means of the non-ICU hospitalized COVID-19 patients' group and ICU hospitalized COVID-19 patients' group. ‡) p-values which represent the comparison between the means of control group and the ICU hospitalized COVID-19 patients' group. All p-values were calculated by unpaired Student's *t* test.

Severe cases of COVID-19 show an abnormality in liver functions

To evaluate the effect of COVID-19 disease on the liver function among hospitalized patients with COVID-19, we compared the levels of ALT (U/L), AST (U/L), and ALP (U/L) among the participants of each two groups separately. Our results showed a significant increase in the mean concentration of ALT (28.92 ± 0.88 U/L) in the non-ICU hospitalized COVID-19 patients compared with the controls (21.77 ± 0.37 U/L, p value < 0.05), (Figure 2A). The results also found that the ICU hospitalized COVID-19 patients showed a more pronounced increase in the levels of ALT (44.82 ± 2.28 U/L) compared with the non-ICU hospitalized COVID-19 patients (28.92 ± 0.88 U/L, p value < 0.05) and the control subjects (21.77 ± 0.37 U/L, p value < 0.05), (Figure 2A). We also found that the mean concentration of AST significantly increased among the ICU hospitalized COVID-19 group (42.41 ± 1.90 U/L) compared with the controls (19.85 ± 0.35 U/L, p value < 0.05) as well as the non-ICU hospitalized COVID-19 group (30.72 ± 0.98 U/L, p value < 0.05), (Figure 2B). Regarding the effect of COVID-19 on ALP levels, there was a significant increase in the concentrations of ALP among the non-ICU hospitalized COVID-19 patients (84.78 ± 2.98 U/L) compared with the controls (50.79 ± 1.13, p value < 0.05), (Figure 2C). Interestingly, our findings also revealed that ICU hospitalized COVID-19 patients had a higher increase in ALP levels (145.30 ± 3.90 U/L) compared to the non-ICU hospitalized COVID-19 patients (84.78 ± 2.98 U/L, p value <0.05) and control

individuals (50.79 ± 1.13, p value < 0.05), respectively (Figure 2C). These findings suggest that COVID-19 disease is associated with abnormal liver function tests and that abnormality increases during COVID-19 severity.

Patients with COVID-19 who are admitted to the ICU have more liver injury

We assessed whether COVID-19 disease may develop liver injury among the hospitalized patients with COVID-19. Liver injury was defined in patients who had raised ALT, AST and/or ALP more than two times the upper limit unit of normal [14]. Our results revealed that there was no patient with liver injury among the non-ICU hospitalized COVID-19 patients as well as the control group (Figure 3). Interestingly, the majority of COVID-19 patients who were admitted to the ICU had liver injury. Based on ALT, AST and ALP levels, we found that 73 (52%), 77 (55%) and 38 (27%) of the ICU hospitalized COVID-19 patients had liver injury, respectively (Figure 3A, B, C). In addition, the results showed that 12 (8.5%) of the ICU hospitalized COVID-19 patients who had liver injury died compared to 5 (3.5%) patients who had abnormal liver test results (Table 1). These findings propose that severe cases of COVID-19 may be associated with more liver injury, which may increase the risk of death.

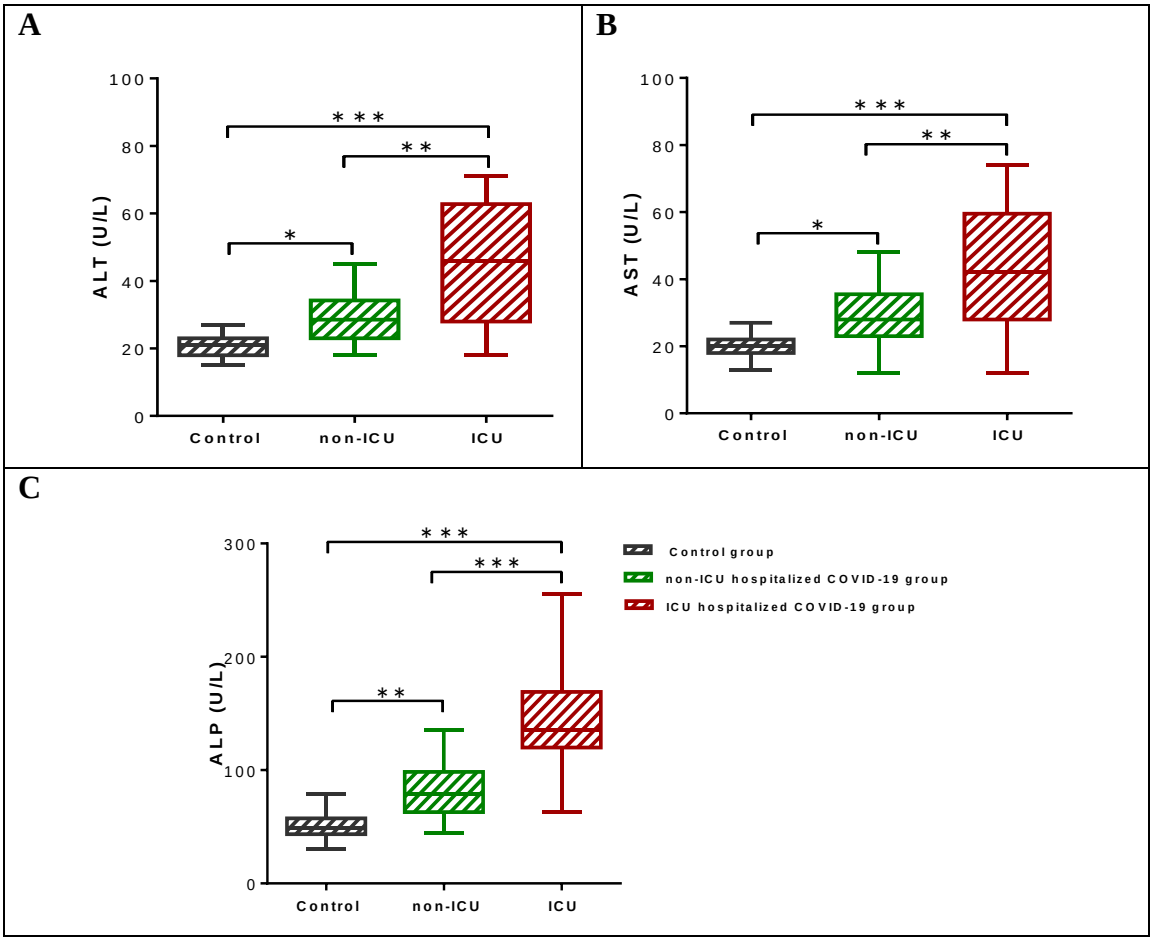

Figure 2: Severe cases of COVID-19 show an abnormality in liver functions.

The levels of ALT (U/L), AST (U/L), and ALP (U/L) were evaluated in the COVID-19 patients as well as controls. (A-C) Box and whisker graphs displaying the concentrations of ALT (U/L), AST (U/L), and ALP (U/L) in the peripheral blood of controls (n=100, black boxes), non-ICU hospitalized COVID-19 patients (n=130, green boxes), and ICU hospitalized COVID-19 patients (n= 140, brown boxes). Statistical analysis was performed using unpaired t-test (*p ≤ 0.05, ** p < 0.01, *** p < 0.001).

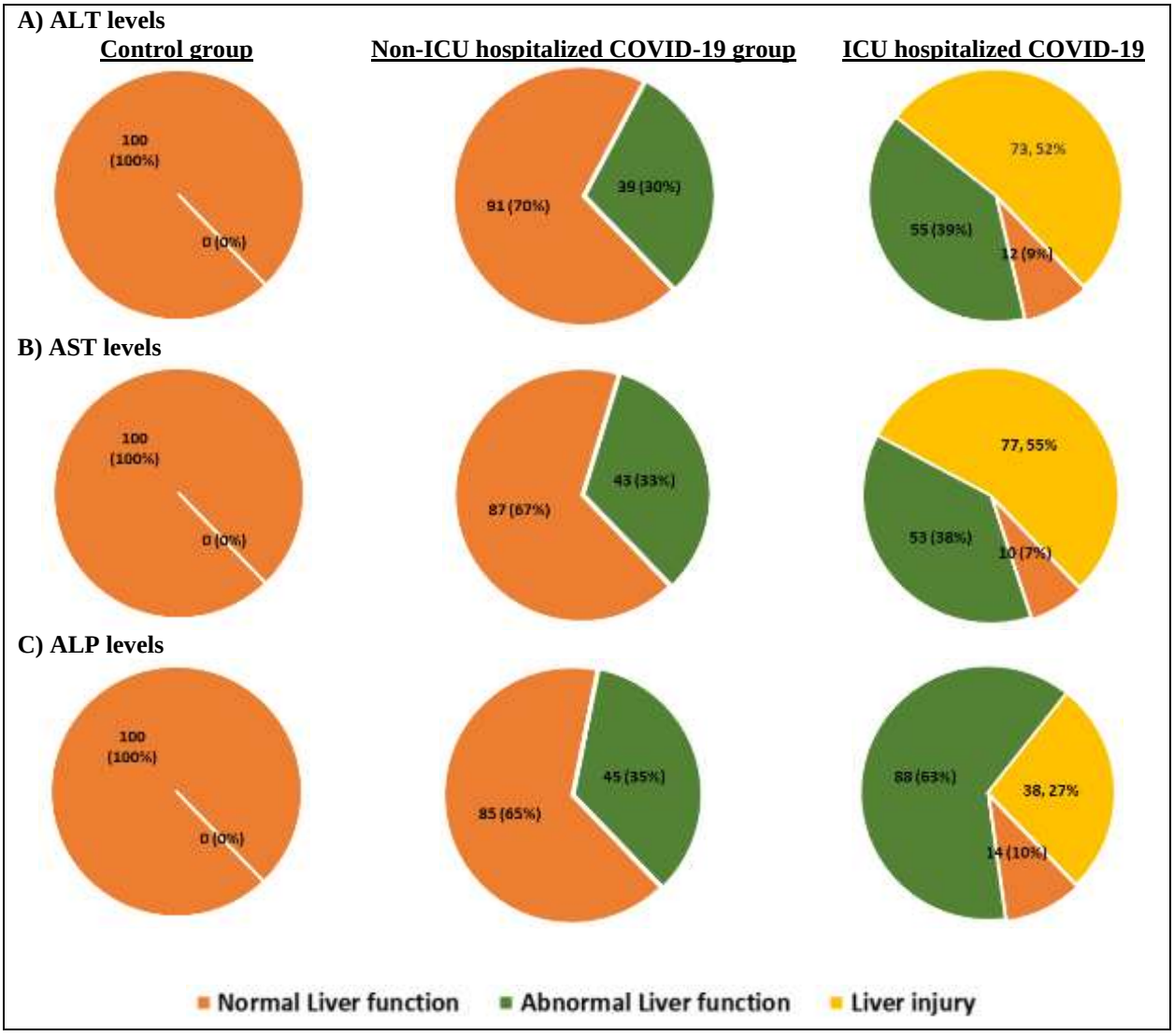

Figure 3: severe cases of COVID-19 are associated with more liver injury.

Liver injury is defined in patients who had raised ALT, AST and/or ALP more than two times the upper limit unit of normal range. (A-C) Pie chart illustrating the percentages of COVID-19 patients as well as the control subjects who had liver injury based on the levels of ALT, AST and ALP, respectively. Data represent the relative proportion (frequency) of the indicated responses measured.

DISCUSSION

In severe cases, COVID-19 patients develop severe lung disease and multi-organ dysfunction, which may increase the risk of death [1,2,15]. One of these organs which may affect by SARS-CoV-2 infection is the liver, causing liver dysfunction [21,22]. High percentage of patients with severe COVID-19 have an abnormal elevation in the liver enzymes as well as liver injury [22–25]. One study showed that 76.3% of the hospitalization COVID-19 patients had abnormal liver function and 21.5% of them with liver injury [14].

The present study was conducted to assess the effect of COVID-19 disease on the liver function among the hospitalized

COVID-19 patients by evaluation the liver enzymes levels (ALT, AST, ALP). Our results showed a significant increase in the levels of the liver enzymes among the hospitalized COVID-19 patients. Of note, we found that the majority of COVID-19 patients who were admitted to the ICU had liver injury. Supporting the findings of our study, several studies assessed the clinical features of COVID-19 patients and the factors that could possibly cause liver injury by SARS-CoV2 infection 26–31. The results of these studies found an increase in the levels of liver enzymes mainly ALT, AST and ALP as well as abnormal liver function tests among high percent of COVID-19 patients. A recent meta-analysis study of 47 studies showed that about 15 - 20% of the hospitalized patients with COVID-19 had abnormal elevations in the liver enzymes, including AST, ALT, and ALP 32. Another study carried out by Cholankeril and his colleagues found that 26 of 65 COVID-19 patients (40%) had abnormal liver enzymes, and 4 of them were noted to have liver injury due to a 2-fold elevation in liver enzymes during their SARS-CoV-2 infection 33. In the same perspective, Yao et al. conducted a study to evaluate the changes in liver function in hospitalized patients with COVID-19 30. Of the 40 cases, there were 21 cases (52.5%) with elevated ALT and 16 cases (40%) with both ALT and AST elevated, and liver damage occurred in 22 of the 40 confirmed patients (55%). Also, they found that the probability of liver injury in critically ill patients was significantly greater than that of non-critically ill patients. However, the results showed that liver injury was more likely to occur in patients who used drugs like lopinavir/ritonavir and methylprednisolone. This suggests that some drugs for COVID-19 may have hepatic toxicity in some patients. In the same context, 148 patients with confirmed COVID-19 were included in a study performed by Fan and his colleagues. At the time of admission to the hospital, 55 (37.2%) of the COVID-19 patients had abnormal liver function 34. According to their findings, a greater proportion of COVID-19 patients with abnormal liver function (57.8%) had received lopinavir/ritonavir treatment after admission 34. In the United States, another retrospective observational cohort study was conducted to investigate liver test abnormalities and their relationship to clinical outcomes in 1,827 hospitalized COVID-19 patients 24. The results showed that patients with COVID-19 had a pronounced increase in liver enzyme levels during hospitalization, and the drugs used for the treatment of COVID-19 (lopinavir/ritonavir, hydroxychloroquine, remdesivir, and tocilizumab) were linked to higher levels of liver enzymes and the development of liver injury. In our study, we did not assess the effect of COVID-19 medication on liver function because there was a missing data about the treatment protocol of COVID-19, and we excluded the patients using hepatotoxic drugs from the study.

Because our study is a retrospective study, we did not follow up the COVID-19 patients after their recovery. However, some studies assessed the longitudinal effects of SARS-CoV-2 infection on liver function [35,36]. In this context, Zhu et al. evaluated the liver function among COVID-19 patients immediately after hospitalization, before discharge and one year after discharge [36]. They found that 32.2% of the COVID-19 patients with abnormal liver function immediately after hospitalization, 45.8% before discharge and 28.8% after one year of discharge. Another study found that among 461 COVID-19 patients, 28.4% of them had liver dysfunction, and there was a marked improvement in liver function after 12 months of discharge where 13% of COVID-19 patients had liver dysfunction and most of them with pre-existing liver disease [35]. These findings suggest that long-term monitoring of liver function is essential mainly among COVID-19 patients with pre-existing liver disease.

This study had some limitations, including a retrospective single-center study design and limited access to laboratory, imaging, and medication variables, which may influence key clinical outcomes. In addition, as with other retrospective studies, there is a possibility of selection bias. Another drawback of our study was the challenge of evaluating overlapping drugs used for the treatment of COVID-19 patients while they are in the hospital. Future studies involving multiple centers with a larger sample size are required to confirm the findings of our study.

In conclusion, our findings added to a growing body of evidence indicating that SARS-CoV-2 infection is associated with a significant proportion of concomitant liver abnormalities that increase during COVID-19 severity, leading to liver injury and increasing the risk of COVID-19 mortality. As the etiology of liver abnormality noted in SARS-CoV-2 infection is not completely understood, further prospective studies are needed to clarify the exact mechanism by which SARS-CoV-2 infection can affect liver function and develop liver injury.

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تأثير كوفيد-19 على إنزيمات الكبد لدى مرضى كوفيد-19 في المستشفيات في قطاع غزة: دراسة بأثر رجعي

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ملخص

في الحالات الشديدة، مرض فيروس كورونا الجديد 2019 (كوفيد-19) يمكن أن يتسبب في فشل الجهاز التنفسي وخلل في العديد من الأعضاء، بما في ذلك إصابة الكبد. قيمت هذه الدراسة تأثير كوفيد-19 على وظائف الكبد بين مرضى كوفيد-19 في المستشفى. تم تقسيم المشاركين (ن=370) إلى ثلاث مجموعات: المجموعة الضابطة (ن=100)، مجموعة كوفيد-19 التي لم تدخل وحدة العناية المركزة (ن=130) ومجموعة كوفيد-19 في وحدة العناية المركزة (ن=140). تم تقييم مستويات إنزيمات الكبد في عينات الدم لكل مشارك. أظهرت نتائجنا زيادة كبيرة في مستويات ALT بين مرضى كوفيد-19 في وحدة العناية المركزة مقارنة مع مرضى كوفيد-19 غير المعالجين بوحدة العناية المركزة (قيمة p اقل من 0.01) وعناصر التحكم (قيمة p اقل من.(0.001 أيضًا تركيز AST زاد بشكل كبير بين مجموعة كوفيد-19 في وحدة العناية المركزة مقارنةً بعناصر التحكم (قيمة p اقل من 0.05) ومجموعة كوفيد-19 غير المعالجة بوحدة العناية المركزة (قيمة p اقل من .(0.01 كان لدى مرضى كوفيد-19 في وحدة العناية المركزة زيادة أعلى في مستويات ALP مقارنة بمرضى كوفيد-19 غير المعالجين بوحدة العناية المركزة والضوابط (قيم p اقل من 0.001). استنادًا إلى مستويات ALT وAST وALP، وجدنا أن 73 (52٪) و77 (55٪) و38 (27٪) من مرضى كوفيد-19 يعانون من إصابة في الكبد. من هؤلاء 12 (8.5٪) ماتوا مقارنة بـ 5 (3.5٪) مرضى يعانون من خلل في وظائف الكبد. تشير هذه النتائج إلى أن مرض كوفيد-19 مرتبط بوظائف الكبد غير الطبيعية وإصابة الكبد.

الكلمات الدالة: كوفيد-19، فيروس كورونا 2 المرتبط بالمتلازمة التنفسية الحادة الشديدة، إنزيمات الكبد، تلف الكبد.

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Ethnobotanical Study of the Most Lamiaceae Used as Medicinal and Culinary Plants by the Population of Bejaia Province, Algeria

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ABSTRACT

This study was carried out to safeguard ancestral knowledge about most Lamiaceae plants used by the population of the Bejaia region (north-eastern Algeria) for medicinal and food purposes. Using 200 questionnaire sheets, ethnobotanical surveys were performed between February and July 2020. The data were analyzed by calculating quantitative indices such as Relative Citation Frequency (RFC), Plant Part Value (PPV) use index, and Fidelity Level (FL). It was shown that women hold ethnobotanical information (52%) more than men (48%), older persons are expected to provide more reliable information and the majority of users have a university level. Otherwise, herbal medicine is used more in rural areas than in urban and 55% of the studied plants are cultivated while 45% are wild. The leaves are the most used part (PPV = 0.592) and the infusion method was the most commonly used (69.7%). Ethnobotanical analysis revealed that *Mentha spicata* L. (RFC=0.44), *Lavendula stoechas* L. (RFC=0.215), and *Salvia officinalis* L. (RFC=0.205) are frequently used. Digestive pathologies are the major therapeutic indications and 41.44% of species were used for seasoning meat and fish. This survey could constitute an important source of information and a database for further research in the fields of phytochemistry and pharmacology.

Keywords: Ethnobotany, Bejaia, medicinal plants, Lamiaceae, therapeutic effects, culinary uses.

Introduction

Various plants were employed by the population since ancient times for their medical and food needs. [1] Nowadays, medicinal plants still play a major role in the treatment of several pathologies, especially in rural areas for financial causes and owing to inconvenience in accession to sanitary concerns. [2, 3]

Ethnopharmacological studies and ethnobotanical surveys constitute a very reliable approach to the exploration of ancestral information and are efficient techniques for determining and documenting medicinal plants. [4] These last have constantly been a substantial constituent of the ancestral method of cure in developing nations, and have also been a basic section of the folklore and cultural customs of regional societies. [5, 6]

In some African nations, equivalent to 90% of the inhabitants depends uniquely on wild herbals as the origins of medications. Furthermore, renewed attention concerning medicinal and food plant research and their ancestral application by various indigenous populations of Africa were noted in actual years. [7]

Algeria is known for its floristic diversity which constitutes a rich phylogenetic source with about 3000

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species belonging to several botanical families. [8, 9] The Kabylie region is characterized by high biodiversity which affords Algeria to hold a prominent position amongst the North African nations, with a large medicinal heritage. [10-12] The use of medicinal plants takes a substantial location in the practices of treatment in Algeria, and the Kabylie location is a factual example.

Despite conventional remedies being acknowledged as prominent to preserve the health of 70-80% of the African population, quite a few comparative works have been achieved on the usage of medicinal plants by several civilizations or ethnic groups in the African continent. The main research works on herbal medicines have been realized in the advanced world. [7] On the other hand, an investigation of the Algerian medicinal literature demonstrates that the information on local medicinal species is incomplete and scattered. It is therefore essential to undertake such investigations to identify the uses by the population of medicinal plants. In addition, because of the excessive cost of drugs, and low income, plants constitute an important source for the population.

Even though the previous study was conducted on some Algerian native plants, there are no documented records of species employed as culinary herbs. In addition, to our knowledge, only one work is devoted solely to the study of three species was carried out in the region of petite Kabylie (Bejaia). [12] So, this survey aims to study and register the ancestral information and usage of plant species for gastronomic aims and their therapeutic prominence for humans in the Bejaia region.

The current study focuses on the achievement of a survey of the main plants belonging to the Lamiaceae family with the inhabitants of the Bejaia Departement (petite Kabylie). This will make it possible to have a collection relating to the ancestral use of these medicinal and food herbs. It is awaited that this research study will emphasize a few prospective herbs for probable huge extent productivity for both culinary and medicinal purposes about the economical rising of the population.

Material and Methods

Plants material

Twelve plants of the Lamiaceae family are selected, namely: horehound (*Ballota nigra* L.), basil (*Ocimum basilicum* L.), lavender (*Lavendula stoechas* L.), apple mint (*Mentha rotundifolia* L.), pennyroyal (*Mentha pulegium* L.), spearmint (*Mentha spicata* L.), lemon balm (*Melissa officinalis* L.), oregano (*Origanum vulgare* L.), brunelle (*Prunella vulgaris* L.), rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), and thyme (*Thymus vulgaris* L.). These plants were selected based on their dual culinary and medicinal uses by the inhabitants of the study region (Bejaia), as well as for their availability in all the studied areas.

Description of the study area

This ethnobotanical survey was carried out in the department of Bejaia (petite Kabylie) (**Figure 1**) which is one of the largest coastal regions of Algeria. The total population of this region is estimated at 978,050 inhabitants in the census of December 2018 in an area of 3261 Km^2.

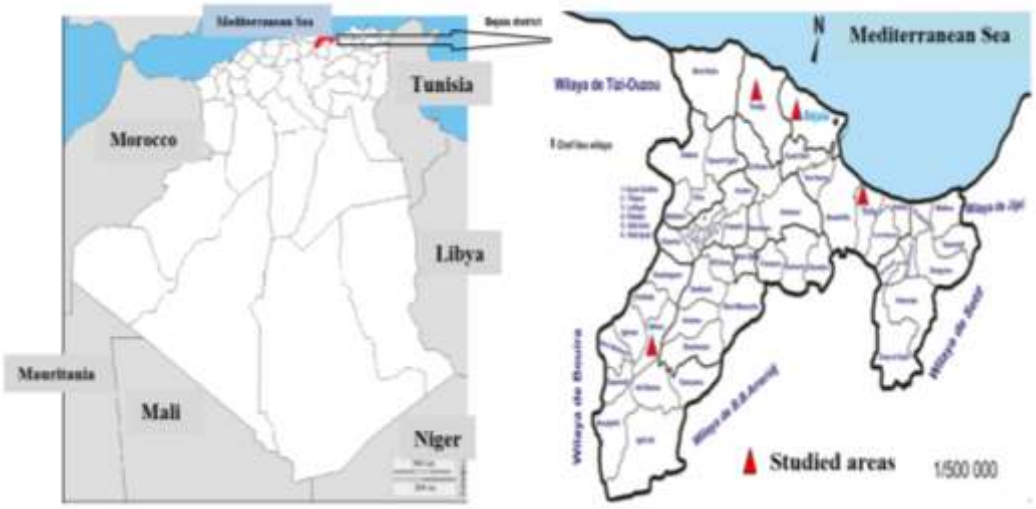

Figure 1. Map of the study areas.

Data collection

To assemble information related to the uses of the selected plants, ethnobotanical surveys were achieved during a period from February to July 2020 in different localities of Bejaia province (Algeria). The characteristics of the different sites were summarized in **Table 1**.

The area was divided into five layers (strata) (S1, S2, S3, S4, and S5) (**Figure 1**), samples of 40 persons constituted each of the 5 strata are arranged jointly to represent 200 informants.

The interviews with the informants were conducted in the Kabyle dialect of the region.

Identification and conservation of plant species

The harvested specimens were identified employing the following book: New flora of Algeria and southern desert regions.[9]

The regional name and the botanical and ethnobotanical features of the studied herbs were written on the specimens placed in the Laboratory of 3BS, Department of Food Sciences, Faculty of Natural Sciences and Life, Bejaia (Algeria).

Data Analysis

Descriptive statistical techniques using Microsoft Excel 2007 allowed the determination of frequencies and percentages which were employed to explore the socio-demographic information of informers.

Examination of ethnobotanical information was performed by the RFC, the PPV use index, the FL, and the Informant Consensus Factor (ICF) as reported by Orch et al. [2] and Sukumaran et al. [5]

Table 1.Characteristics of the different study areas.

Study sites	Geographical coordinates	Altitude (m)	Area(km^2)	Average precipitation (mm)	Avearge T (°C)	Climate	Number of inhabitants
Akbou	Latitude : 36.45, Longitude : 4.55° 27′ 0″ N, 4° 33′ 0″ E	Minimal:180 Maximal: 400 Average : 290	52.2	358.6	26.2	Mediterranean with dry summer	190 766
Toudja	Latitude : 36.76, Longitude : 4.89°45′31″ N, 4°53′36″ E	Minimal : 252 Maximal:1317 Average : 785	16713	672	19	A warm Mediterranean climate with dry summer	10 534

Study sites	Geographical coordinates	Altitude (m)	Area(km^2)	Average precipitation (mm)	Avearge T (°C)	Climate	Number of inhabitants
Tichy	Latitude: 36.6675, Longitude : 5.1600936° 40′ 3″ N, 5° 9′ 36″ E	Minimal : 0 Maximal : 435 Average : 22	56.7	600	15.8	Mediterranean with hot summer	16 546
Bejaia city	Latitude : 36.75, Longitude : 5.07 36° 45′ 0″ N, 5° 4′ 0″ E	Minimal : 1 Maximal : 660 Average : 331	120.2	739	22.2	Warm and temperate Mediterranean climate	190 766
Tazmalt	Latitude : 36.39, Longitude : 4.39° 23′ 4″ N, 4° 23′ 57″ E	Minimal : 100 Maximal : 100 Average : 100	33.6	672.3	27.5	Mediterranean with dry summer	30 968

Relative frequency of citation (RFC) (0 < RFC < 1) is determined to evaluate the regional relevance of each plant and is estimated as follows :

$RFC = Fc/N$

Fc: citation frequency

N: number of respondents

Plant Part Value (PPV)

The PPV is measured to estimate the relevance of each employed part of the species by the responders, it is calculated as follows :

$PPV = RUplant\ part/RU$

with RUplant part is the sum of reported uses per part of the plant.

RU= the number of reported uses of all parts of the plant.

Fidelity level (FL)

To calculate the most frequently employed plant for treating a specific illness type by the informers of the Bejaia zone, the fidelity level (FL) was determined as follows:

$FL = Np/N \times 100$

with Np is the number of use-reports cited for a specific plant for a distinct disease type and N is the total number of usage statements mentioned for any afforded species.

Informant consensus factor (ICF)

ICF was calculated for each category of ailments to identify the agreements of the informants on the reported cures for the group of ailments. It corresponds to the number of use citations in each category (n_{ur}) minus the number of species used (n_t), divided by the number of use citations in each category minus one :

$ICF = n_{ur} - n_t / n_{ur} - 1$

Results and discussion

Sociodemographic profile of the respondents

During the ethnobotanical survey carried out in the five chosen strata (**Table 2**), we found that both genders (men and women) are interested in traditional medicine with little benefit going to women (52%) (**Table 3**). Commonly women are more concerned with herbal treatment and the preparation of recipes based on medicinal plants. In Algeria, in general, and in the wilaya of Bejaia in particular, it is the women who cook and use medicinal plants more to heal their families.

Table 2. Distribution of the surveys by strata.

Strata	Names of Strata	Number of Inquiries
Strata 1	Akbou (Chellata, Ighrem)	40
Strata 2	Toudja (Tala hiba, Ifrene)	40
Strata 3	Tichy (Tizi Ahmed, Lemaâden)	40
Strata 4	Bejaia city (Centre-ville, Ihaddaden)	40
Strata 5	Tazmalt (Laâzib, Aït Lhadj)	40
Total respondents		**200**

These results are consistent with those found in other ethnobotanical works performed in different provinces of Algeria. [10, 12-14] Likewise, in other countries, several studies have found that women hold ethnobotanical information more than men. [2, 15-17]

This is probably because women are traditionally the custodians of the secrets of medicinal plants. [15] It is also well known that the women take care of the preparation of the recipes for their care and those of their children. [16]

The utilization of the species chosen in this survey interests different age categories of the study areas. According to the results, the old people where those over 60 years constitute 25% followed by the age groups of [41-60], <20, [31- 40], and [20-30] with 22.5%, 21%, 18.5%, and 13%, respectively. The elderly informants are the better connoisseurs in the field of phytotherapy since they have a long experience accumulated and transmitted from one generation to another. We also noted that young people, including those under the age of 20, are interested in herbal medicine and this can be explained by the fact that they consult the Internet to adopt traditional remedies. Similar results have been obtained by several other studies at a national scale. [10, 18]

We remarked that the surveyed people with different levels are interested in traditional medicine. Most of the participants have a university level (27.5%), followed by those with a secondary level (25%), and middle school (21%). Nonetheless, a low percentage was attributed to people with primary education (1.5%). Meanwhile, the percentage of illiterate people is not negligible (25%) **(Table 3**). These results showed that it is not only illiterate people who are interested in herbal medicine but also educated people.

Table 3. Socio-demographic characteristics of the participants in the study area.

	Distribution	Number of informants	Percentages (%)
Gender	Male	96	48
	Female	104	52
Age groups	<20	42	21
	[20-30]	26	13
	[31-40]	37	18.5
	[41-60]	45	22.5
	>60	50	25
Educational level	Illiterate	50	25
	Primary	3	1.5
	Middle	42	21
	Secondary	50	25
	University	55	27.5
Habitat	Town	40	20
	Village	160	80

Our results are consistent with those achieved by Zahir, Elazaoui, Chakouri and Naouer [19] in Beni Mellal - Khénifra region (Morocco) who found that 18.09% of herbal medicine users are illiterate, while 81.91% correspond to different educational levels.

As regards the living environment of the people questioned, those who live in rural areas tend to use herbal medicine with a percentage of 80%. Zahir, Elazaoui, Chakouri and Naouer [19] demonstrated similar results in the Beni Mellal-Khénifra region of Morocco where most of the respondents come from the villages and mountains since rural participants are the main consumers of medicinal plants. This may be justified by the fact that the rural population maintains good contact with nature. Besides, most of the inhabitants of rural areas have a low income which does not allow them to seek medical help and/ or buy medicines which leave them in

front of herbal medicine as a cheaper remedy. [19]

Relative Frequency of Citation

The number of citations of the studied species in the different study areas varies from 14 to 88 times. The most cited species was *Mentha spicata* L. with an RFC of 0.44, followed by *Lavendula steochas* L. (RFC = 0.215), *Salvia officinalis* L. (RFC=0.205), *Thymus vulgaris* L. (RFC=0.2), *Origanum vulgare* L. (RFC= 0.195), *Mentha pulegium* L. (RFC=0.175), *Ocimum basilicum* L. (RFC= 0.17), *Ballota nigra* L. (RFC =0.14), *Melissa officinalis* L. as well as *Prunella vulgaris* L. (RFC= 0.135), then *Rosmarinus officinalis* (RFC= 0.09), and finally *Mentha rotundifolia* L. (RFC= 0.055) **(Table 4)**.

Table 4. List of the studied plants from the Lamiceae family in the study area.

Scientific name	Local name	Parts used	Preparation mode	Administration	Fc	RFC
Ballota nigra L.	Amarnouy	Aerial part, leaves	Infusion	Internal	28	0.14
Lavendula stoechas L.	Amezir	Flowering tops, leaves	Infusion, Decoction		43	0.215
Mentha rotundifolia L.	Timejja	Leaves	Infusion, Decoction, Pression	External	14	0.055
Mentha pulegium L.	Flewou	Aerial part, leaves	Infusion, Decoction	Internal	35	0.175
Mentha spicata L.	Nââna	Leaves	Infusion	Internal	88	0.44
Melissa officinalis L.	Ifer t'Zizoua	Leaves	Infusion	Internal	27	0.135
Ocimum basilicum L.	Lehvak	Leaves	Infusion, Pression	Internal External	34	0.17
Origanum vulgare L.	Zaâther	Leaves, tiges	Infusion, Decoction	Internal	39	0.195
Prunella vulgaris L.		Fruits, leaves, tige	Pression, Decoction	Internal	39	0.195
*Rosmarinus officinalis*L.	Amezir Ouamen	Leaves, flowers	Infusion, Decoction	Internal	18	0.09
Salvia officinalis L.	Agurim, Tazzurt	Leaves, stems	Decoction, Infusion, Pression, Maceration	Internal	41	0.205
Thymus vulgaris L.	Thizaathrin	Aerial part, leaves	Infusion, Decoction	Internal	40	0.2

M. spicata and *O. basilicum* are widely used as herbal medicine and to season food since they are available throughout the year in both rural and urban regions of Bejaia. The 10 other medicinal species are also cited more than 10 times which implies that they are also used by the inhabitants of the study areas.

Frequency of use of the plants according to their origin

According to the plants' origin, 110 (55%) stated that the majority of the studied species are cultivated and 90 (45%) answered that they were wild while no person (0%) indicated that they were imported. Chehma, Djebar, Hadjaiji and Rouabeh [20] has shown that a rate of 58% of herbal medicine in south-eastern Algeria is wild species.

In connection to the harvest period, the obtained results revealed that 28.4% of respondents stated that the plants studied are perennials whatever the climatic conditions, 43.19% indicated that these medicinal plants are more harvested during the spring period, and 26.47% mentioned that their harvest is done in summer. Conversely, 0.78%, and 1.17% reported that the plants are harvested in autumn and winter, respectively **(Table 5).**

These findings are close to the results of the ethnobotanical study conducted in Ouargla region (northern Sahara in Algeria) where informants denote that 72% of herbal species are collected in spring. [20]

Parts used

With the intent to make curative preparation, several parts (leaves, flowers, fruit, seed...) can be used depending on the plant's nature. The determination of the Plant Part Value (PPV) use index demonstrated that the leaves are the most employed (PPV= 0.592), come then the fruits (PPV= 0.160), the stems (PPV=0.118), the aerial parts (PPV=0.097), the roots (PPV=0.021), and finally the seeds (PPV= 0.010) **(Figure 2)**. This divergence in the proportions of the plant parts exploited can be justified by

the type of plants studied which are aromatic and the volatile oils will be concentrated in the leaves. In addition, this can be explained by the easiness of collecting the leaves. [2] The dominance of the leaves used has been corroborated by other surveys performed in other localities of Algeria. [10, 21, 22]

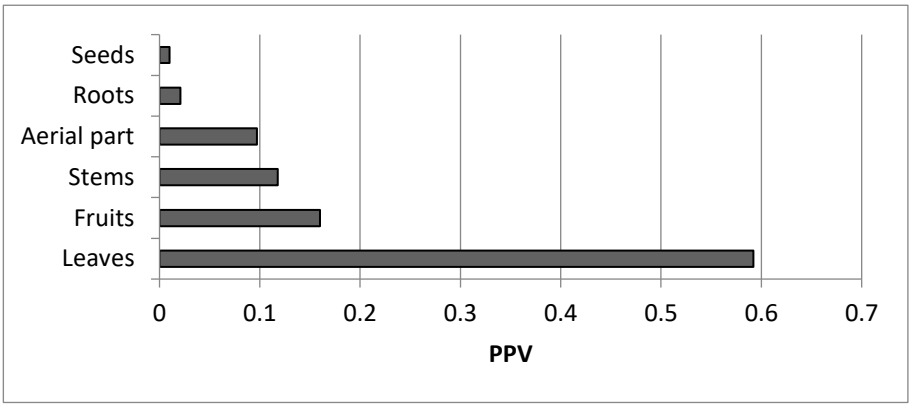

Figure 2. Percentages of different parts used for the treatment of differnt diseases in the zone of study.

Method of preparation and administration

Depending on the preparation method, infusion is the most employed (69.67%) followed by decoction (18.03%), pressing (7.38%), and maceration (4.92%) (**Table 5**). The best preparation method for plants would preserve all of their properties while allowing the extraction and assimilation of the active ingredients. [23]

These results are in agreement with those found by other Algerian researchers [20, 21] who recorded the dominance of the infusion mode with percentages of 50% and 20.45%, respectively.

Various modes were utilized in the administration of herbal preparations. The oral route contributed 77.33% of the total species, followed by an external application (14.67%), and inhalation of smoke (8%) (**Table 5**).

The oral application is popular as to the finding of Benarba [22] and Khaled-Khodja, Brahmi, Madani and Boulekbache-Makhlouf [12] who reported it as the leading route of application used in East (Bejaia), and in South-West Algeria (Saharian regions). It is also in agreement with the result of various ethnobotanical studies conducted elsewhere in Morocco [16, 24] and indicates that the oral way is the predominant route of application.

Table 5. Data related to the plants studied.

	Distribution	**Number of informants**	**Percentages (%)**
Plant type	Cultivated	110	55
	Wild	90	45
	Imported	0	0
Harvest period	Perennials	73	28.4
	Spring	111	43.19
	Summer	68	26.47
	Autumn	2	0.78
	Winter	3	1.17
Part used	Leaves	46	16.03
	Stems	34	11.85
	Aerial part	28	9.76
	Roots	6	2.09
	Seeds	3	1.05

	Distribution	Number of informants	Percentages (%)
Preparation mode	Infusion	170	69.67
	Decoction	44	18.03
	Pressing	18	7.38
	Maceration	12	4.92
Administration mode	Orally	174	77.33
	External application	33	14.67
	Inhalation	18	8
Reason for use herbal medicine	Low cost	169	45.43
	Effectiveness	161	43.28
	Better than conventional drugs	22	5.91

The provenance of knowledge of medicinal plants

Ailments treated by studied plants

Plant medicines are employed for several diseases in Algeria. The studied species are most frequently used to treat affections of the digestive system (78%) and affections of the annex glands of the digestive tract (30%), respiratory disorders (60%), neurological disturbances (39.5%), dermatological affections (13%), genitourinary disorders (7.5%), and cardiovascular diseases (6.5%). This was also confirmed by the highest ICF value (1.81) attributed to gastrointestinal and related diseases (**Table 6**).

Some species were registered as being employed for more than one ailment. Among the 12 species studied, the highest FL of 64.28% was noticed for *Mentha rotundifolia*, followed by *Prunella vulgaris* L. (48.14%), and *Ballota nigra* L. (46.42%). *Rosmarinus officinalis* L., *Melissa officinalis* L. and *Ocimum basilicum* L. have the same FL of almost 44%. On the other hand, 7 of the plants had the lowest FL value of about 20% **(Table 6)**.

Table 6. Distribution of the different therapeutic uses of medicinal plants, Informant Consensus Factor (ICF) and Fidelity Level (FL) values for common medicinal plants used by local traditional healers by ailment category.

Ailment categories	Use citations	%	ICF value	Most preferred species with specific ailment	FL (%)
Dermatological infections/ diseases	26	13	0.96	*Prunella vulgaris* L. (stops bleeding, oral infections)	**48.14**
				Salvia officinalis L. (wounds, mouth, throat, canker sores, palate, gingivitis care)	**24.39**
Gastro-intestinal ailments	156	78	0.95	*Mentha rotundifolia* L. (stomachic, increased appetite)	**64.28**
				Ballota nigra L. (digestive, anti-vomiting, anti-diarrhea)	**46.42**
				Rosmarinus officinalis L. (anti-diarrhea, liver, vesicles care)	**44.44**
Affections of the annex glands of the digestive tract	60	30	0.86	*Melissa officinalis* L. (vomiting during pregnancy, promotes bile secretion, promotes digestion)	**44.44**
				Ocimum basilicum L. (nausea, vomiting)	**44.11**
				Origanum vulgare L. (digestive problems, improves appetite)	**33.33**
				Salvia officinalis L. (diarrhea and ulcer)	**31.70**
				Thymus vulgaris L. (gastroenteritis, stimulates digestion)	**22.5**
				Mentha spicata L. (carminative, stomachic, abdominal pain, appetizer, nausea, vomiting)	**20.45**
Respiratory system diseases	120	60	0.95	*Origanum vulgare* L. (respiratory, cold)	**46.15**
				Thymus vulgaris L. (asthma, bronchitis, cold, flu)	**37.5**
				Prunella vulgaris L. (cold, lung care)	**37.03**
				Mentha spicata L. (cold, flu)	**36.36**
				Mentha pulegium L. (respiratory problems)	**34.28**
				Lavendula stoechas L. (release the respiratory tracts, respiratory expectorant, respiratory system infections)	**30.23**
				Salvia officinalis L. (calm the smoker's cough)	**26.82**
Circulatory system/ cardiovascular diseases	13	6.5	1	*Ocimum basilicum* L.	**32.35**

Ailment categories	Use citations	%	ICF value	Most preferred species with specific ailment	FL (%)
Genio-urinary ailments	15	7.5	0.86	*Rosmarinus officinalis* L. (fertility, diuretic)	**38.88**
				Thymus vulgaris L. (painful periods, inflammation of the bladder)	**20**
				Salvia officinalis L.(regulates the cycle, painful periods)	
Neurological disorders	79	39.5	0.95	*Melissa officinalis* L. (against anxiety)	**44.44**
				Ballota nigra L. (sedative, sleep inducer, anxiety, nervous disorders)	**39.28**
				Mentha spicata L. (insomnia, calm nerves, headache, against dizziness, relaxing)	**30.68**
				Lavendula stoechas L. (dizziness, migraine, nervous sleep disturbances)	**20.93**
				Rosmarinus officinalis L. (dizziness, migraine, calm anxiety)	**20.90**

Evidently, the remedies for usually indicated sicknesses have the highest FL values and those species less preferable for the treatment of particular sicknesses have low FL values. Plants with high FL might be an evidence of their potency effects on a particular sickness. In this survey, the FL values indicate that the Bejaia population prefers some species from the Lamiaceae family for treating certain diseases.

It is quite clear that the majority of species (9) are used to treat gastrointestinal ailments and affections of the annex glands of the digestive tract. *Mentha rotundifolia* L. with the highest FL value was recommended as stomachic and increased appetite and four species (*Ballota nigra* L., *Melissa officinalis* L., *Ocimum basilicum* L., *Mentha spicata* L.) were used as anti-vomiting. Moreover, four species (*Ballota nigra* L., *Rosmarinus officinalis* L., *Ocimum basilicum* L., *Salvia officinalis* L. were useful as anti-diarrhea.

Many species (8) with high FL values were also indicated in respiratory system diseases mainly *Origanum vulgare* L., *Thymus vulgaris* L., *Prunella vulgaris* L., and *Mentha spicata* L. which were preconised particularly in cold cases. In addition, five species are effective in neurological disturbances mainly *Melissa officinalis* L. which was considered as a remedy for anxiety.

It is well known that many species of the Lamiaceae family are effective in the treatment of gastrointestinal diseases in many countries. The main diseases treated with plants from Algeria based on the results of the European project RUBIA, that research has been achieved on the traditional use and handling of plant species in seven Mediterranean countries (Albania, Algeria, Cyprus, Egypt, Italy, Morocco, and Spain) ; are stomach-ache and the majority of identified Algerian plants are also used as a sedative. [25] The same trend was revealed by Benarba [22] where gastrointestinal illnesses were the most commonly treated pathologies with medicinal plants in southwest Algeria (33.6%), followed by respiratory (23%) and cardiovascular diseases (9%).

The dominance of treatment of digestive disorders by medicinal plants of the Lamiaceae family has been reported in several studies. Indeed, *O. basilicum* as well as *M. spicata* are considered as a natural remedy to treat digestive disorders in the region of Settat (Morocco). [26] Miara et al. [27] confirmed that *L. steochas*, *R. officinalis*, and *M. pulegium* are used to treat stomach aches in the region of Tiaret (Algeria). Additionally, *M. officinalis*, *M. puleguim*, *T. vulgaris*, and *O. vulgare* are exploited by the population of the Talassemtane region (Western Rif of Morocco) to treat digestive ailments. [28] On the other hand, Kemassi et al. [29] stated in their study that *M. rotundifolia* is used in the region of Rabat (Morocco) to treat osteo-articular disorders. As for *S. officinalis*, it is recognized as a remedy for diabetes in the M'Zab valley (northern eastern Algerian Sahara). [30] *M. pulegium* was considered as the most effective species for treating pathologies related to the respiratory system. [25]

Among the species from Kabylie (Tizi-Ouzou department) investigated by Meddour and Meddour-Sahar[11] to heal disturbances of the digestive tract, *B. nigra*, *M. pulegium*, *M. spicata*, *M. rotundifolia*, *O. vulgare* and *L.*

stoechas displayed principal place and are effective in several gastrointestinal disorders (indigestion, constipation, diarrhea, dysentery, stomach ulcer, hyperacidity, vomiting, bloating, intestinal worms).

In the southwest of Algeria, *Thymus vulgaris* L., *Mentha pulegium* L., and *Ocimum basilicum* L. are mostly used in the management of respiratory-related ailments. [22]

Apart from the major indications in table 6, the people questioned indicated that the majority of plants are also used in the form of herbal tea to relieve painful periods (basil, sage, oregano, spearmint, pennyroyal). In addition, it has been indicated that basil is recommended for eye disease (conjunctivitis) and is used in feverish states, fatigue and to reduce fever. Apple mint and oregano are indicated for headaches and as pain relievers and anesthetics. Lavender is used for liver and gallbladder disorders, dizziness, thinness, eczema, psoriasis, acne, nervous sleep disorder and as an antidiabetic. Pennyroyal is recommended for stomach aches, to address fertility problems in women, and to lower cholesterol levels and headaches. The brunelle is used to treat oral infections and sore throats and it is febrifuge. Thyme is used as an antiseptic, against insect bites, and for inflammation of the bladder. Lemon balm is considered an antiviral and can be used for dental pain. Spearmint is investigated as a diuretic, helps to eliminate kidney stones, and relieves stings associated with insect bites. Oregano is indicated as an insecticide, antiseptic and is used for migraine and toothache. Sage activates circulatory functions and is recommended in cases of physical and intellectual overwork.

Reasons for the use of herbal medicine

In our studies, 45.43% of the interviewees used herbal medicine since they had a low socio-economic level, 43.28% have chosen them for their efficiency and, 5.91% believe that they have fewer side effects than drugs (**Table 5**). These findings coincide with those found in most ethnobotanical studies made in neighboring countries like Morocco. [2, 31]

Various plants are used for their therapeutic properties by populations around the world, following historical, cultural, and economic considerations. The conventional health system remains very expensive for several populations in the world. In addition, phytotherapy treatment is one of the scarce therapeutic tools allowing a symptomatic and truly etiological approach to be taken. [1, 2]

Culinary uses

We report that the majority of respondents (92 responses out of 222) indicated that the studied species were used for seasoning and flavouring meat and fish; this can be explained by the fact that the plants chosen are mainly aromatics. They were employed also to season pasta, herbal teas, soups, sauces, and salads with percentages of 22.52, 15.77, 13.06 f, 14, 6.31, and 0.9%, respectively (**Figure 3).**

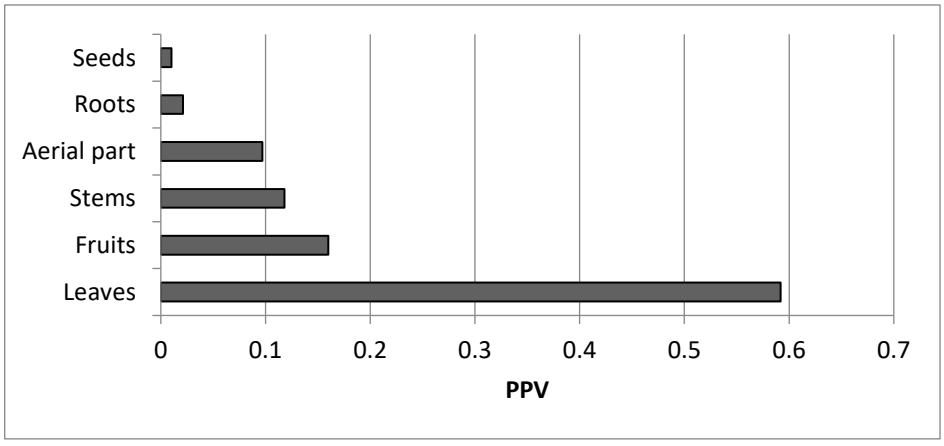

Figure 3. Culinary use of the studied plants.

According to Derridj, Ghemouri, Meddour and Meddour-Sahar [10] not less than 42 species (34.21% of the total plants) from Kabylie (Tizi-Ouzou Department) have food applications. They are used to prepare some very famous dishes in the region of Kabylie such as wild tea (Letaï lekhla), spice bread (Aghrum lehwal), steamed dishes (Aâmouch), and special pasta with vegetables (Avazine).

D'Antuono and Elementi [32] reported that herbs from the Lamiaceae family possess the main properties of spices, including antioxidant and antimicrobial, and are used as a food flavoring and as a preservative. Extracts of rosemary, sage, thyme, oregano, and mixtures of several plants of the Lamiaceae (such as provincial herbs) are commonly used by the population. The extracts of these species are available in many forms for seasoning foods such as sauces, dressings, ready-made pasta, and meat.

CONCLUSION

This survey is the first to explore the knowledge of the most medicinal and culinary herbs from the Lamiaceae family by the population of Bejaia province (north-eastern Algeria) to demonstrate their pharmacological effects and food value. The prevalence of usage of medicinal herbs in this department is nearly related to the profile of the persons interrogated. Hence, young persons, likened to the aged, typically do not recognize the names and utility of most plant species. Women and men have a common medicinal awareness, with a minor discrepancy in the proportion of medicinal herb utilization among the two genders, with little benefit going to women. Among the plants explored *Mentha spicata* L. (spearmint), *Lavendula stoechas* L. (lavender), and *Salvia officinalis* L. (sage), are the most used and have more applications. The data procured allowed us to display that the majority of species in the survey region are extensively employed to treat affections of the digestive system, respiratory disorders, and neurological disturbances. As culinary herbs, they are adopted mostly for seasoning and flavouring meat and fish. These applications are principally dealt with leaves, and with the infusion. Finally, it occurs from this study that the ancestral usage of medicinal herbs yet perseveres in the explored department, although the advancement in the medical field.

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Conflicts of interest

All authors stated that there was no conflict of interest regarding the study design and publication of the manuscript.

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دراسة عرقية نباتية لمعظم الشّفويّات المستخدمة كنباتات طبية وفي طهي من قبل سكان ولاية بجاية، الجزائر

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ملخص

أجريت هذه الدراسة لحماية معرفة الأجداد حول معظم نباتات الشّفويات الّتي يستخدمها سكان مدينة بجاية (شمال شرق الجزائر) للأغراض الطبية والغذائية. باستخدام 200 ورقة استبيان، تم اجراء دراسات استقصائية عرقية نباتية بين فبراير ويوليو2020. تم تحليل البيانات خلال حساب المؤشرات الكمية مثل تكرار الاقتباس النّسبي (RFC)، ومؤشر استخدام قيمة جزء النّبات (PPV)، ومستوى الإخلاص (FL). وتبين أن النّساء يحتفظن بمعلومات نباتية عرقية (52%) أكثر من الرجال (48%)، ومن المتوقع أن يقدم كبار السّن معلومات أكثر موثوقية وأن غالبية المستخدمين لديهم مستوى جامعي. بخلاف ذلك، يتم استخدام الأدوية العشبية في المناطق الريفية أكثر من المناطق الحضرية ويتم زراعة 55% من النّباتات المدروسة بينما 45 % منها برية. الأوراق هي الجزء الأكثر استخداما (PPV=0.592) وكانت طريقة التسريب هي الأكثر استخداما (69.7%). أظهر التحليل العرقي أن *Mentha spicata* L. (RFC=0.44)، *Lavendula stoechas* L. (RFC=0.215)، و *Salvia officinalis* L. (RFC=0 .205) مستخدمة بشكل متكرر. أمراض الجهاز الهضمي هي المؤشرات العلاجية الرئيسية و41.44% من الأنواع استخدمت لتتبيل اللحوم والأسماك يمكن أن يشكل هذا الاستطلاع مصدرا مهما للمعلومات وقاعدة بيانات لمزيد من البحث في مجالات الكيمياء النّباتية وعلم العقاقير.

الكلمات الدالة: علم النبات العرقي، بجاية؛ نباتات طبية، الشّفويات، تأثيرات علاجية، استخدامات في الطهي.

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Development and Validation of a Simple and Sensitive ICP-MS Method for the Quantification of Elemental Impurities in Propafenone Hydrochloride Drug Substance

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ABSTRACT

Elemental impurities are substances present in drug products, excipients, or drug formulations. They may be formed by the presence of catalysts and environmental contaminants. Elemental impurities can be detected by a sophisticated method such as Inductively Coupled Plasma Mass Spectrometry (ICP–MS). ICP-MS is an advanced method to detect elemental impurities in drug substances. In this study Propafenone hydrochloride drug was used, Propafenone Hydrochloride is an antiarrhythmic medication belonging to class 1C used to prevent supraventricular and ventricular arrhythmias. The present study was aimed to develop and validate inductively coupled plasma mass spectroscopic (ICP–MS) method for detection of elemental contaminants, i.e., Class 1, Cd, Pb, As, Class 2A, Hg, Co, V, and Class 2B impurities such as Ni, T1, Se, Ag, Au, Pd, Ir, Os, Rh, Ru, and Pt. Total 17 elemental impurities were detected in Propafenone Hydrochloride and this method was employed for the regular sample analysis of 17 elemental impurities in Propafenone Hydrochloride for pharmaceutical use. The instrument conditions were set using RF power of 1550 W, auxiliary gas of 0.5 L/min, and nebulizer flow of 1.01 L/min nebulizer pump pressure was 0.10 rps, spray chamber temperature was 2°C, and mode used was He, He flow rate was 4.3 mL/min and the energy discrimination rate was 3.0 V. The technique is sensitive and may identify desirable elemental impurities within permissible regulatory limits when additional elements are present. The proposed ICP-MS approach has been found to be accurate, precise, linear, rugged, robust, and convenient for the quality control of the drug substance propafenone hydrochloride. The linearity results for each impurity were 0.9990. The methods were validated according to USP requirements and International Council for Harmonization ICH guidelines. The suggested approach is an excellent quality control tool for the concurrent quantitative assessment and detection of elemental contaminants at low levels in the drug substance propafenone hydrochloride.

Keywords: Elemental Impurities, ICP-MS, Propafenone Hydrochloride, Validation.

INTRODUCTION

Elemental impurities are substances found in pharmaceuticals, excipients, and drug formulations. They may be produced as a result of the presence of one or more catalysts and environmental contaminants. These impurities can occur naturally or may be intentionally introduced. Interactions with equipment and containers can produce these impurities.[1] The chemical entity of Propafenone hydrochloride is 1-[2-[2-hydroxy-3-(propylamino) propoxy] phenyl]-3-phenylpropan-1-one. Fig 1 It is an antiarrhythmic medication of class 1C that is used to prevent supraventricular and ventricular arrhythmias. It also has anaesthetic properties on a local level. It works as an anti-arrhythmic agent. It contains propafenone (1+). It is a very effective

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antiarrhythmic medication for ventricular arrhythmias. Additionally, its beta-blocking effects are modest.[2]

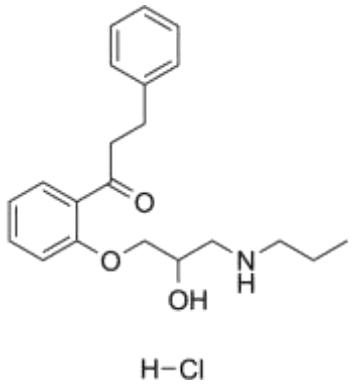

Fig 1 Chemical Structure of Propafenone Hydrochloride

Elemental impurities are categorized into Class 1, 2A & 2B impurities. Class 1 elements are proven human toxins with little to no application in the development of drugs. Their inclusion in drug products often results from elements that are widely used. These four components should be evaluated due to the inherent hazards they carry. Class 1 impurities include As, Cd, Hg, and Pb.[3]

Due to the relatively high possibility of Class 2A elements which may be present in the pharmaceutical drug products, it is necessary to assess their risk across all potential sources of elemental impurities and administration routes. Impurities that are specified in Class 2A include Co, Ni, and V. Class 2B elements have a lower possibility of being in the therapeutic product due to their low abundance and limited possibilities for co-isolation with other materials. Therefore, they can be excluded from the risk evaluation unless they are purposefully included during the production of drug ingredients, excipients, or other parts of the drug product. Ag, Au, Ir, Os, Pd, Pt, Rh, Ru, Se, and TI are class 2B impurities.[4,5]

Few elements such as arsenic, cadmium, mercury and lead are known to produce toxic effects in humans (often through a variety of different mechanisms), and are therefore should be measured to estimate exposure. ICP-MS has several advantages, it is a multi-element technique with a broad analytical range and a low detection limit, it has high sample throughput, a low sample volume, it requires little sample preparation and it has high resolution. From a laboratory point of view, ICP-MS method has many advantages over other methods perhaps the most significant advantage of is its multi-element analysis, it can measure multiple elements simultaneously in a single analysis. Coupled with simple sample preparation and short analysis time, very high sample throughput is the major advantage of ICP-MS in the laboratory.[6]

USP 232_ establishes PDE limits for a variety of inorganic (elemental) impurities, including Cd, Pb, As, Hg, Co, V, Ni, T1, Se, Ag, Au, Pd, Ir, Os, Rh, Ru, and Pt. The recommended maximum daily dose and daily exposure limits for elemental impurities must be scaled for the drug under investigations, so for a substance with a daily dose of 10 g, the elemental impurity level in the dosage form must be less than ten times than the limits shown. Modern instrumental techniques like ICP-MS, which are listed in USP 233, make it simple to measure the requisite limits directly. Currently, the management of elemental impurities in pharmaceutical products is changing from management based on concentrations in drug product components to management based on permissible daily exposures in drug products.[7-9] The developed method was validated according to International Council for Harmonization (ICH) (Q2A) guidelines.[10]

Few methods were developed by RP-HPLC and LC-MS according to literatures, A method was performed using HPLC equipped with a conventional octadecylsilyl silica column and ultraviolet detector Simultaneous determination of serum propafenone and its metabolites.[11] A reversed phase HPLC method was developed to stereoselectively determine enantiomers of propafenone in human plasma.[12] A simple, rapid, accurate, precise, robust and reproducible reverse phase high performance liquid chromatographic method was developed for the determination of Propafenone HCl in pure drug and pharmaceutical dosage form.[13] A simple, precise and accurate RP-UFLC method was developed for determination

of propafenone hydrochloride.[14] A simple, sensitive and rapid High performance liquid chromatography/positive ion electrospray tandem mass spectrometry method was to be developed and validated for quantification of propafenone (PPF) and its two major metabolite 5-hydroxy propafenone (5-OHP) and N-depropyl propafenone (NDP) in human plasma.[15] Another LC–MS/MS method was adopted to develop a novel sample preparation Hybrid SPE phospholipid technology to extract plasma samples for improved phospholipid removal.[16] A rapid spectrophotometric and chromatographic method was developed for the estimation of propafenone hydrochloride in tablet dosage form by Quality by Design (QbD) approach as per ICH Q8(R2) guidelines.[17] In another study a rapid and sensitive LC-MS/MS method was developed and validated for the quantification of propafenone (PPF) and its active metabolite 5-hydroxypropafenone (5-OHP) in human plasma.[18] In this study, a novel method for determining 17 elemental impurities in propafenone hydrochloride was developed and validated by ICP-MS.

MATERIALS AND METHODS

Chemicals and Reagents

The chemicals used were obtained from the following suppliers: Conc. Nitric acid (69%) obtained from (Honey well Trace analysis), Conc. Hydrochloric acid (35%) procured from (Fisher scientific Trace analysis), and Tuning solutions used were procured from (Inorganic ventures) ICP-MS grade. The standards such as Cadmium, Lead, Arsenic, Mercury, Cobalt, Vanadium, Nickel, Thallium, Palladium, Iridium, Osmium, Rhodium, Ruthenium, Selenium, Silver, Platinum were procured from (Inorganic Venture) and Gold standard belonging to (Acc standard). Propafenone Hydrochloride was obtained as a gift sample from Pharmazell India P. LTD

Instruments used

An Agilent ICP-MS 7800 series, an Analytical Balance Radwag AS 82/220.X2, Micropipette Brand 20µL, 200µL, 1000µL and a Microwave Digester Milestone ETHOSUP 17092516 was used for the study. Table 1 Illustrates Instrument parameters

Plasma Condition

The RF Power was 1550 W, the RF matching was found to be 1.80 V, the sample depth was 8.0 mm, nebulizer gas flow was streamed at 1.01 L/min, nebulizer pump pressure was 0.10 rps, spray chamber temperature was 2°C, and mode used was He, He flow rate was 4.3 mL/min and the energy discrimination rate was 3.0 V.

Acquisition Parameters

The acquisition mode was spectrum mode, the peak pattern was 3 points, 3 replicates were used for the study, the sweeps/replicate was found to be 100, the integration time/mass (sec) was observed in the range of 0.0999 sec and the number of masses used was 17.

Tuning solution:

The standard solution was prepared by taking 1 ppb mixture of Li, Y, Tl, Co & Ce as tuning solutions, the expected m/z was found in the range of 7, 89, 205, 58 & 140 for all the five tuning solutions and the % RSD limit was observed to be NMT 15 % for (Li, Y, Tl / Co).

Table 1 Instrument parameters

Parameter	Method Condition	Parameter	Method Condition
Plasma Condition		**Octopole Condition**	
RF Power	1550 W	Energy discrimination	3.0 V
RF Matching	1.80 V	**Acquisition Parameters**	
Sample Depth	8.0 mm	Acq Mode	Spectrum
Nebulizer Gas	flow 1.01 L/min	Peak Pattern	3 points
Nebulizer Pump	0.10 rps	Replicates	Sweeps/Replicate 100
Spray Pattern temperature	2°C	Sweeps/Replicate	100
Mode	He	Integration time mass	0.0999 sec
He Flow	On	Number of masses	17
He flow rate	0.43 ml/min		

Table 2 Specification Limit for the elements

Elements Class	Name of the impurity	Specification Limit in (ppm)	Elements Class	Name of the impurity	Specification Limit in (ppm)
Class - 1	Cadmium	NMT 0.5	Class – 2B	Thallium	NMT 0.8
	Lead	NMT 0.5		Gold	NMT 10
	Arsenic	NMT 1.5		Palladium	NMT 10
Class – 2A	Mercury	NMT 3		Iridium	NMT 10
	Cobalt	NMT 5		Osmium	NMT 10
	Vanadium	NMT 10		Rhodium	NMT 10
	Nickel	NMT 20		Ruthenium	NMT 10
				Selenium	NMT 15
				Silver	NMT 15
				Platinum	NMT 10

Preparation of Diluent:

The diluent solution was assessed by transferring 20 ml of concentrated nitric acid (69%) and concentrated hydrochloric acid (35%) into a 1000 mL volumetric flask, previously rendered with 500 mL of purified water and the volume was adjusted up to the mark with purified water.

Preparation of Standard mix stock solution:

The required volume of standard concentration 1000 ppm of each element were pipetted out and was transferred into a 50 mL volumetric flask and the volume was made up to the level with diluent and mixed well.

Preparation of Standard linearity level solutions:

The required volume of standard was prepared and pipetted out and transferred individually into a separate five 10 mL volumetric flask and 0.2 ml sulfuric acid solution and 4 ml reverse Aquaregia was added the volume was increased up to the required volume with water and mixed well. The required concentrations were pipetted out respectively in parts per billion and labeled as calibration standard level -2 to 6.

Preparation of sample solution

The homogenized sample of about 0.2 g was exactly weighed and transferred into a clean and dried Microwave digestion 50 mL capacity sample vessel and 0.5 mL of concentrated sulphuric acid (98%) was added and the vessels were closed and kept inside Microwave digester following program condition.

Microwave Digestion Program

The vessels were cooled at room temperature after completion of pre-digestion. Then 9.0 ml of concentrated nitric acid and 1 mL concentrated hydrochloric acid was added, then the vessels were closed and kept again for pre digestion for 15 minutes on bench top, then the vessels were kept inside Microwave digester following program conditions.

The vessels were cooled after completion of digestion at room temperature and the sample solution was transferred into a 25 mL volumetric flask, the vessels were washed with a portion of 10 mL purified water and transferred to above volumetric flasks then the volume was adjusted to the mark with purified water and mixed well.

System suitability

A study was conducted to demonstrate the system precision, blank and calibration standard solutions were prepared as per the test method and aspirated into ICPMS system. Correlation coefficient of calibration curve should be ≥ 0.99 for each analyte. Concentration of each analyte in bracketing standard should not be vary by ± 20% of actual concentration.

Specificity

A study was conducted to demonstrate the blank, sample blank, calibration standard solutions and non-spiked test solution as per method of analysis which were prepared and aspirated into ICPMS system. Elements

response was evaluated and the interference of blank and sample blank in each element abundance was calculated. The average (five times aspiration) of each calibration blank CPS and sample blank CPS for each analyte should not be more than 5% of 100% level standard solution CPS.

Determination of LOD and LOQ

A study was conducted to demonstrate the limit of detection and limit of Quantitation level based on the Residual standard deviation method by aspirating six levels (5%, 10%, 25%, 50%, 75% and 100%) with respect to the target level. The LOD and LOQ for each analyte should not be more than 30% of the specification limit. The % RSD of class – 1, class – 2A & 2B elements response at LOQ level should be NMT 20%.

Method Precision

The method precision of test method was evaluated by analyzing six spiked test samples and aspirated into ICPMS system. The content of elemental impurities in sample was calculated. The % RSD of the content of each elemental impurities in six samples should be NMT 20%.

Linearity and Range

To demonstrate the linearity of test method, prepared the standard solutions of LOQ, 25%, 50%,100%, 200% and 300% of the targeted concentration and analyzed as per the method. Correlation coefficient for each analyte should not be less than 0.99.

Ruggedness (Intermediate Precision)

The intermediate precision of test method was evaluated by analyzing six spiked samples and aspirated into ICPMS system. The study was performed on different day and different analyst. The content of elemental impurities in sample was calculated. The % RSD of the content of each elemental impurities in six samples should be NMT 20%. The cumulative % RSD for residue of class – 1, class – 2A & 2Belements in twelve preparations (i.e. method and intermediate precision) for each analyte should not be more than 25%.

Accuracy (Recovery)

To demonstrate the accuracy of test method, recovery of element from spiked samples was evaluated. Samples were prepared by spiking the element class – 1, class – 2A & 2B with sample at different levels ranging from LOQ to 300 % of the target concentration of known standards. The sample solutions were prepared in triplicate at LOQ, 100%, 200% and 300% spiked levels and subtract the content from the unspiked sample. The mean % recovery for each analyte at each level should be 70 % to 130 %.

Robustness

The robustness of the analytical method was established by its reliability against deliberate changes in instrumental condition and sample preparation. The test sample was prepared and spiked at specification level and analyzed as per method of analysis by changing the following parameters, such as variation in stabilization time (± 10%), variation in sample diluent concentration (± 10%),

RESULTS AND DISCUSSION

System suitability

A study was performed to investigate the system precision, blank and calibration standard solutions. The samples were prepared as per the test method and aspirated into ICPMS system. The system suitability parameters were calculated and found to be within the prescribed percentage limits. The correlation coefficient of calibration curve for each analyte was found to be > 0.99. Concentration of each analyte in bracketing standard was within the acceptance criteria (± 20% of actual concentration) from the obtained data it was concluded that system was suitable. [19] The results are summarized in Table–3

Table – 3 System suitability results of Elemental Impurities

System suitability parameter	Correlation coefficient of calibration curve	System suitability parameter	Correlation coefficient of calibration curve
Element Name	**Observed value**	**Element Name**	**Observed value**
Vanadium (V)	17	Cadmium (Cd)	3
Cobalt (Co)	2	Osmium (Os)	1
Nickel (Ni)	2	Iridium (Ir)	1
Arsenic (As)	0	Platinum (Pt)	1
Selenium (Se)	1	Gold (Au)	1
Ruthenium (Ru)	0	Mercury (Hg)	0
Rhodium (Rh)	0	Thallium (Tl)	0
Palladium (Pd)	1	Lead (Pb)	3
Silver (Ag)	11		

Specificity

A study was performed in order to demonstrate the blank, sample blank, calibration standard solutions, and unspiked test solutions which were prepared according to the method of analysis and aspirated into the ICPMS system. The response of the elements was evaluated and calculated, and the interference of the blank and sample blank in each element abundance was noted. The average (five times aspiration) of each calibration blank CPS and sample blank CPS for each analyte should not be more than 5% of 100% level standard solution CPS. From the observed data it was concluded that method was specific. [20] The results are summarized in Table – 4

Table – 4 Specificity results

Element name	% Interference of Blank (Difference from 100% level standard CPS)		Element name	% Interference of Blank (Difference from 100% level standard CPS)	
Vanadium (V)	1.4	1.5	Cadmium (Cd)	0.2	0.8
Cobalt (Co)	0.0	0.1	Osmium (Os)	0.7	0.4
Nickel (Ni)	0.2	0.9	Iridium (Ir)	0.3	0.1
Arsenic (As)	1.2	1.2	Platinum (Pt)	0.1	0.1
Selenium (Se)	0.2	0.2	Gold (Au)	2.2	0.9
Ruthenium (Ru)	0.0	0.0	Mercury (Hg)	0.5	0.2
Rhodium (Rh)	0.0	0.0	Thallium (Tl)	2.1	0.9
Palladium (Pd)	0.1	0.0	Lead (Pb)	1.0	4.3
Silver (Ag)	0.1	0.1			

Determination of LOD and LOQ

A study was carried out to demonstrate the limit of detection and limit of Quantitation level based on the relative standard deviation method by aspirating six levels (5%, 10%, 25%, 50%, 75% and 100%) with respect to the target level. The concentration of LOD and LOQ of the solution was derived by using the formula and the results are evaluated in Table – 5. Mass spectrum was evaluated for the limit of detection and limit of Quantitation level. The LOD level was confirmed by aspirating the solution in triplicate and the precision was determined at limit of Quantitation level by injecting six times a solution of spiked standard with the concentration at LOQ level and calculated the relative standard deviation of peak response. The LOQ for each analyte should not be more than 30 % of the specification limit. LOQ for each analyte was found to be below 30% of the specification limit. The response of LOD solution for each element was found to be consistent. From the LOQ data the % RSD of class – 1, class – 2A & 2B elements response at LOQ level was found to be within 20%. Based on the observed data, it was concluded that the LOD and LOQ value for each elemental impurities reported values were observed to be at lowest possible level. [21]

Table 5-Establishment of LOD and LOQ level of elemental impurity

Element name	Correlationcoefficient	Observed LOD in ppm	Observed LOQ in ppm
Vanadium (V)	0.99996	0.00093	0.00282
Cobalt (Co)	0.99998	0.00038	0.00116
Nickel (Ni)	0.99995	0.00222	0.00673
Arsenic (As)	0.99976	0.00037	0.00111
Selenium (Se)	0.99991	0.00227	0.00689
Ruthenium (Ru)	0.99998	0.00076	0.00230
Rhodium (Rh)	0.99999	0.00053	0.00161
Palladium (Pd)	0.99997	0.00085	0.00257
Silver (Ag)	0.99991	0.00226	0.00686
Cadmium (Cd)	0.99998	0.00004	0.00011
Osmium (Os)	0.99999	0.00054	0.00164
Iridium (Ir)	0.99997	0.00084	0.00255
Platinum (Pt)	0.99993	0.00135	0.00409
Gold (Au)	0.99932	0.00411	0.01245

Method Precision

The method precision of test method was evaluated by analyzing six spiked test samples and aspirated into ICPMS system. The content of elemental impurities in sample was calculated. The relative standard deviations of six sample preparation of each % elemental impurity were found to be within acceptance criteria. The % RSD of the content of each elemental impurities in six samples were found to be less than 20 %. From the obtained data it was concluded that method was precise. [22] The results are summarized in Table –6

Table – 6 Method Precision results

Element name	Recovered concentration in %							% RSD
	Met. Precision-1	Met. Precision-2	Met. Precision-3	Met. Precision-4	Met. Precision-5	Met. Precision-6	Average	
Vanadium	107.7	105.7	109.4	108.2	107.3	105.1	107.2	1.5
Cobalt	94.8	92.7	95.7	94.5	94.4	92.1	94.0	1.4
Nickel	94.4	93.0	95.6	94.6	94.4	92.3	94.1	1.3
Arsenic	94.5	89.3	93.1	94.2	92.3	93.2	92.8	2.0
Selenium	92.7	93.3	98.8	96.3	93.2	91.7	94.3	2.8
Ruthenium	94.1	92.9	95.4	94.4	94.5	92.5	94.0	1.1
Rhodium	94.9	93.1	96.1	95.1	95.0	92.9	94.5	1.3
Palladium	94.1	92.4	94.3	93.8	94.9	93.2	93.8	0.9
Silver	85.8	84.1	88	86.6	87.9	84.9	86.2	1.8
Cadmium	97.0	92.5	94.4	94.5	93.7	91.6	94.0	2.0
Osmium	94.2	95.6	98.6	97.9	98.4	96.6	96.9	1.8
Iridium	97.3	96.0	99.8	98.2	98.8	97.0	97.9	1.4
Platinum	95.9	94.9	97.6	96.6	97.4	94.8	96.2	1.3
Gold	91.1	92.6	100.5	96.4	101.7	98.8	96.9	4.4
Mercury	95.6	94.8	98.4	97.3	97.5	95.7	96.6	1.4
Thallium	91.6	93.1	100.3	95.8	100.9	98.5	96.7	4.0
Lead	94.3	93.0	95.7	94.9	94.6	93.0	94.3	1.1

Linearity and Range

The linearity of test method was demonstrated by preparing the standard solutions of LOQ, 25%, 50%, 100%, 200% and 300% of the targeted concentration and analyzed as per the method. The correlation coefficient was observed to be within the acceptance limit. The Correlation coefficient for each analyte was found to be >0.99. The residual sum of square, the intercept and the slope of the regression line were reported. Based on the linearity, precision and accuracy data, the range of the test method was from LOQ to 300 % of the target concentration was observed to be within the range. From the obtained values, it was concluded that method was found to be linear. [23] The results are summarized in Table –7

Table – 7 Linearity and Range results

Element name	Correlation coefficient	Squared correlation Coefficient (r^2) for linearity levels	% Variation of bracketing standard solution	
			Before linearity levels	After linearity levels
Vanadium	0.99809	0.994	10	4
Cobalt	0.99996	0.999	0	4
Nickel	0.99992	0.999	1	5
Arsenic	0.99989	0.999	5	8
Selenium	0.99993	0.999	2	3
Ruthenium	0.99997	0.999	0	6
Rhodium	0.99992	0.999	3	1
Palladium	0.99997	0.999	1	5
Silver	0.99894	0.997	3	1
Cadmium	0.99989	0.999	4	2

Element name	Correlation coefficient	Squared correlation Coefficient (r^2) for linearity levels	% Variation of bracketing standard solution	
			Before linearity levels	After linearity levels
Osmium	0.99993	0.999	2	9
Iridium	0.99995	0.999	4	0
Platinum	0.99996	0.999	4	0
Gold	0.99999	1.000	1	12
Mercury	0.99957	0.998	3	2
Thallium	0.99903	0.997	2	11
Lead	0.99994	0.999	3	2

Ruggedness

The intermediate precision of test method was evaluated by analyzing six spiked samples and aspirated into ICPMS system. The study was performed on different day and different analyst. The content of elemental impurities in the sample was calculated. The relative standard deviations of six sample preparation of each % elemental impurity were found to be within acceptance criteria. The % RSD of the content of each elemental impurities in six samples were found to be below 20 %. The cumulative % RSD for residue of class – 1, class – 2A & 2B elements (i.e. method and intermediate precision) for each analyte was found to be less than 25 %. From the obtained data it was concluded that the method was precise and rugged. [24] The results are summarized in Table 8.

Table –8 Ruggedness results

Element name	Recovered concentration in %							% RSD
	Int. Precision-1	Int. Precision-2	Int. Precision-3	Int. Precision-4	Int. Precision-5	Int. Precision-6	Average	
Vanadium	116.7	117.7	117.2	115.7	118.6	115.8	117.0	1.0
Cobalt	102.0	102.4	102.1	101.4	103.3	101.5	102.1	0.7
Nickel	100.8	101.5	101.2	100.8	102.6	101.0	101.3	0.7
Arsenic	98.1	102.8	98.0	101.0	103.2	99.1	100.4	2.3
Selenium	101.0	101.5	98.8	101.8	101.8	101.3	101.0	1.1
Ruthenium	101.2	101.6	101.1	100.9	102.8	100.9	101.4	0.7
Rhodium	101.3	102.1	101.2	101.4	102.5	101.8	101.7	0.5
Palladium	100.7	100.1	101.1	98.7	102.4	102.0	100.8	1.3
Silver	89.4	89.7	91.1	90.0	95.2	91.7	91.2	2.4
Cadmium	103.5	98.9	100.5	99.1	102.8	102.3	101.2	1.9
Osmium	98.3	101.3	100.4	100.7	103.7	101.6	101.0	1.7
Iridium	101.6	102.5	103.0	102.1	104.4	103.2	102.8	1.0
Platinum	100.1	101.4	101.2	100.5	102.9	101.0	101.2	1.0
Gold	92.0	95.9	101.3	95.7	105.4	103.7	99.0	5.3
Mercury	98.7	101.6	101.8	100.2	102.7	99.9	100.8	1.5
Thallium	94.6	98.4	102.4	97.4	106.7	103.9	100.6	4.5
Lead	98.5	99.6	98.6	98.3	100.0	98.7	99.0	0.7

Accuracy/ Recovery

To demonstrate the accuracy of test method, recovery of element from spiked samples was evaluated. Samples were prepared by spiking the element class – 1, class – 2A & 2B with sample at different levels ranging from LOQ to 300 % of the target concentration of known standards. The sample solutions were prepared in triplicate at LOQ, 100 %, 200 % and 300 % spiked levels and subtract the content from the unspiked sample. The mean % recovery for each analyte at each level was found to be within 70 % to 130 %. From the above data, it was concluded that method was accurate. [25] The results are summarized in Table – 9

Table –9 Accuracy/ Recovery results

Name of the elements	LOQ level Recovered conc. in ppm			100%level Recovered conc. in ppm		
	1	2	3	1	2	3
Vanadium (V)	0.0033	0.0033	0.0033	0.0887	0.0887	0.0884
Cobalt (Co)	0.0012	0.0012	0.0012	0.0382	0.0386	0.0381
Nickel (Ni)	0.0067	0.0068	0.0069	0.1531	0.1545	0.1519
Arsenic (As)	0.0011	0.0011	0.0011	0.0113	0.0112	0.0114
Selenium (Se)	0.0064	0.0067	0.0067	0.1157	0.1147	0.1163
Ruthenium (Ru)	0.0022	0.0022	0.0021	0.0762	0.0762	0.0759
Rhodium (Rh)	0.0015	0.0015	0.0015	0.0772	0.0771	0.0764
Palladium (Pd)	0.0023	0.0023	0.0023	0.0755	0.0754	0.0746
Silver (Ag)	0.0067	0.0069	0.0069	0.1086	0.1031	0.1109
Cadmium (Cd)	0.0001	0.0001	0.0001	0.0038	0.0038	0.0040
Osmium (Os)	0.0016	0.0015	0.0014	0.0744	0.0742	0.0747
Iridium (Ir)	0.0026	0.0025	0.0024	0.0773	0.0779	0.0768
Platinum (Pt)	0.0038	0.0038	0.0037	0.0772	0.0768	0.0763
Gold (Au)	0.0112	0.0111	0.0115	0.0680	0.0696	0.0749
Mercury (Hg)	0.0007	0.0007	0.0007	0.0233	0.0231	0.0230
Thallium (Tl)	0.0008	0.0007	0.0007	0.0056	0.0055	0.0060
Lead (Pb)	0.0002	0.0002	0.0002	0.0039	0.0040	0.0040

Robustness

Effect of variation in Stabilization time

The robustness of the analytical method was established by its reliability against deliberate changes in instrumental condition and sample preparation. The test sample was prepared and spiked at specification level and analyzed as per method of analysis by changing the following, variation in stabilization time (± 10%). The system suitability parameters were evaluated by calculating the % RSD of the content of each elemental impurity in the sample as per the variant test method. The % RSD of the content of each elemental impurities in duplicate spiked samples were found to be below 20 %. From the data presented, it was observed that method was robust and precise. [26] The results are summarized in Table – 10

Table – 10 Robustness results

Robustness – Actual condition							
Element name	**Correlation coefficient**	**Robust-1 % recovered conc.**	**Robust-2 % recovered conc.**	**Average**	**% RSD**	**% Variation of Bkt. std solution recovery before sample**	**% Variation of Bkt. std solution recovery after sample**
Vanadium	0.9929	114.8	116.9	115.9	1.3	14	15
Cobalt	0.9998	100.3	102.5	101.4	1.5	0	1
Nickel	0.9998	100.6	101.6	101.1	0.7	2	0
Arsenic	0.9998	96.9	100.4	98.7	2.5	1	1
Selenium	0.9995	100.5	103.6	102.1	2.1	4	0
Ruthenium	0.9999	101.1	101.6	101.4	0.3	1	0
Rhodium	0.9998	101.3	101.8	101.6	0.3	1	0
Palladium	0.9999	100.9	101.0	101.0	0.1	1	2
Silver	0.9998	89.4	90.3	89.9	0.7	12	14
Cadmium	0.9998	102.6	100.6	101.6	1.4	0	3
Osmium	0.9999	98.6	102.1	100.4	2.5	1	0
Iridium	0.9998	102.8	103.8	103.3	0.7	2	3
Platinum	0.9998	101.2	101.8	101.5	0.4	0	1
Gold	0.9997	94.0	99.3	96.7	3.9	0	0
Mercury	0.9999	100.8	102.5	101.7	1.2	1	0
Thallium	0.9984	96.0	101.0	98.5	3.6	2	3
Lead	0.9996	101.0	100.7	100.9	0.2	3	2

Method variation details

The method variations were performed by taking the actual condition, the stabilization time was 50 seconds, the lower variation limit was found to be (-10%) 45 sec and the Higher variation (+10%) was observed at 55 seconds. The calibration data of elements are summarized in Table 11 Fig 2, 3 & 4.

Table 11 Calibration data of Elements

Calibration of Elements								
Vanadium	**Cobalt**	**Nickel**	**Arsenic**	**Selenium**	**Ruthenium**	**Rhodium**	**Palladium**	**Silver**
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
22.112	9.672	39.127	2.88	28.127	19.186	19.618	18.8	26.5
45.981	20.241	81.228	5.816	60.488	40.215	41.24	39.729	60.937
91.276	40.172	160.111	11.707	119.185	79.843	80.782	79.059	118.794
115.582	60.372	240.324	18.048	181.045	120.05	120.364	120.554	179.271
155.917	79.616	319.503	24.171	239.736	160.089	159.074	160.273	241.353
	Cadmium	**Osmium**	**Iridium**	**Platinum**	**Gold**	**Mercury**	**Thallium**	**Lead**
	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	0.986	18.556	19.481	19.227	16.98	5.891	1.381	0.96
	2.004	39.449	41.187	40.741	39.022	12.258	3.173	2.028
	3.996	79.24	80.068	80.384	78.231	23.92	6.387	4.013
	6.046	119.859	119.863	120.251	121.768	35.963	9.941	6.005
	7.968	160.804	159.837	159.531	160.18	48.017	13.107	7.988

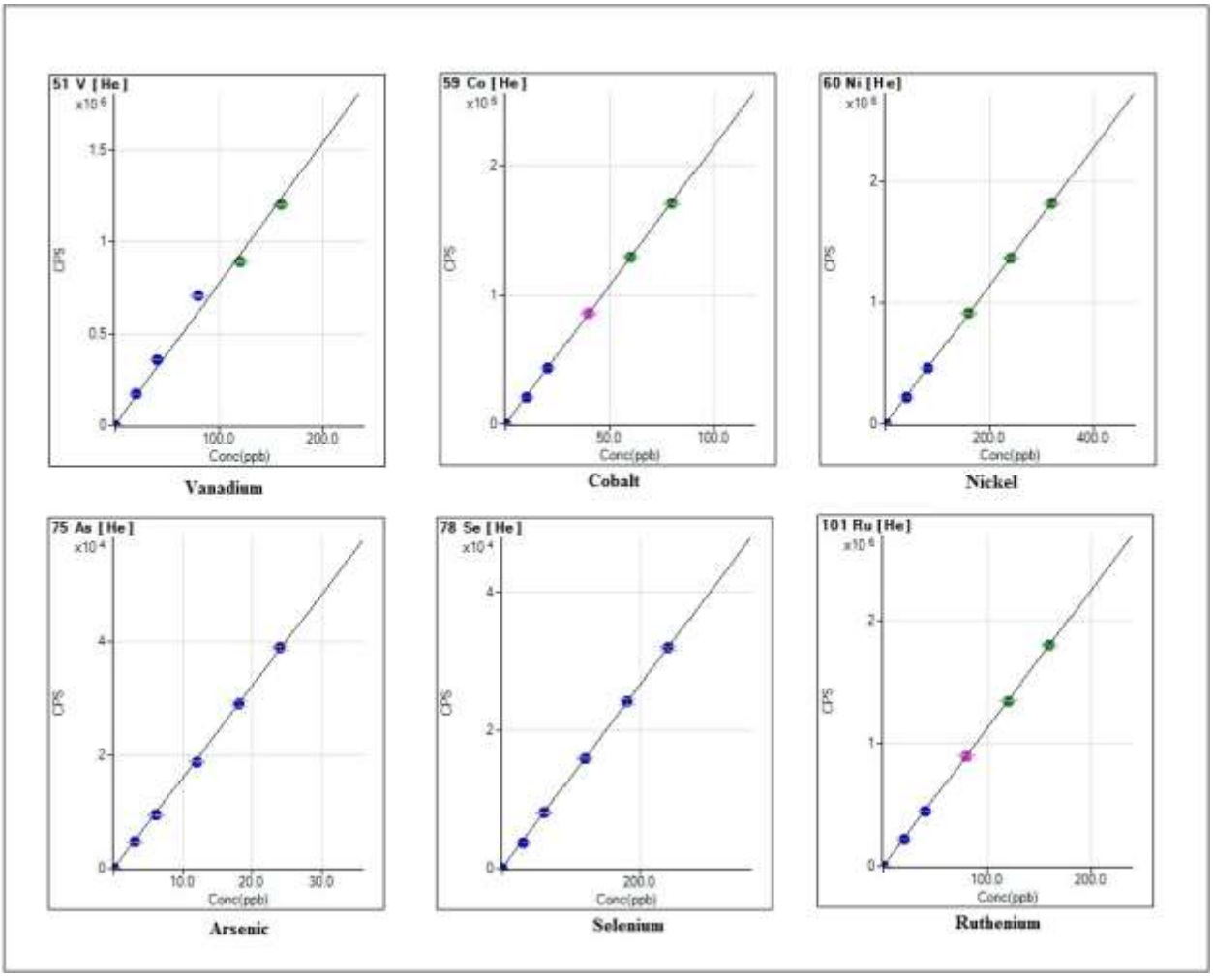

Fig 2 Calibration data for V, Co, Ni, As, Se and Ru

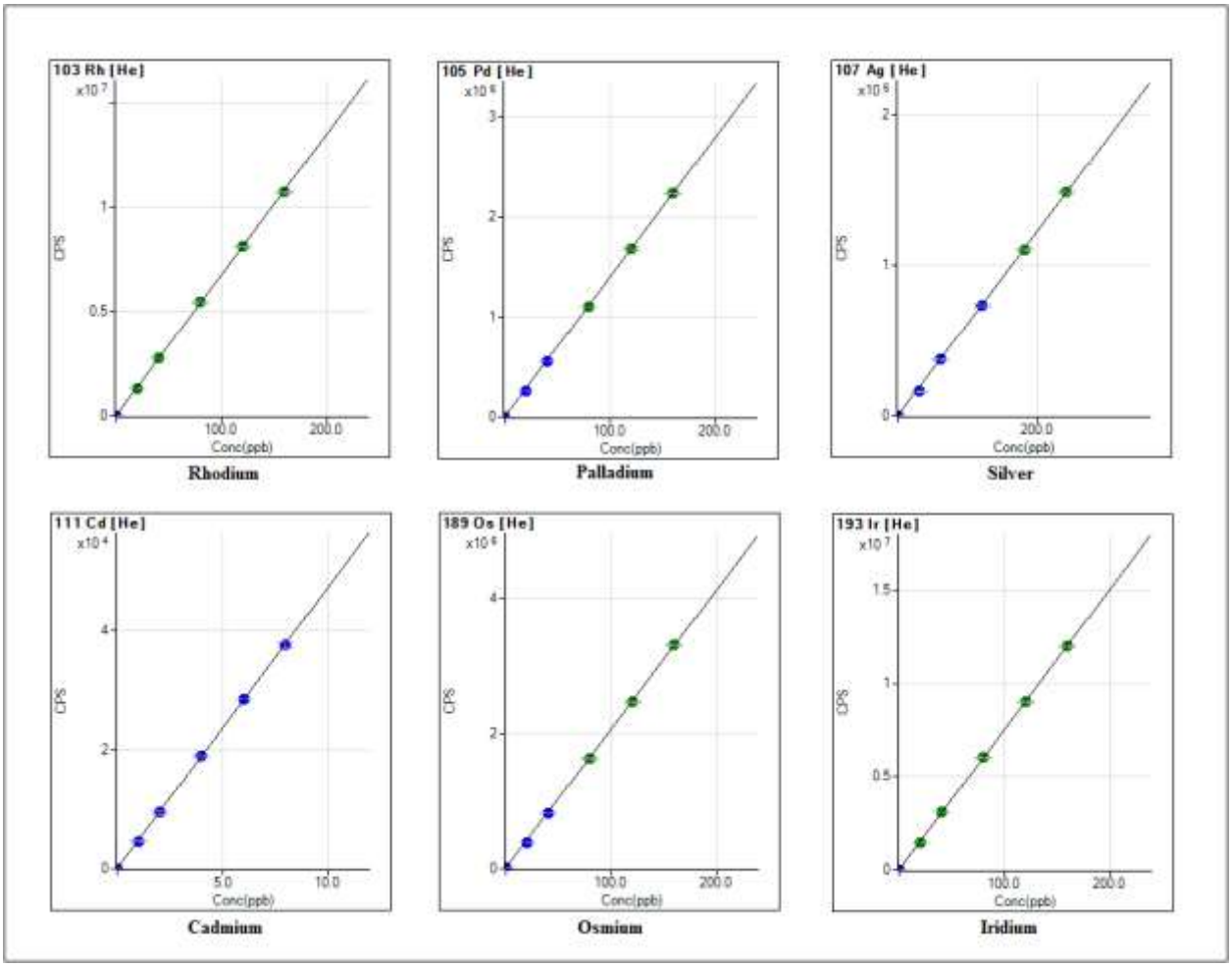

Fig 3 Calibration data for Rh, Pd, Ag, Cd, Os, Ir

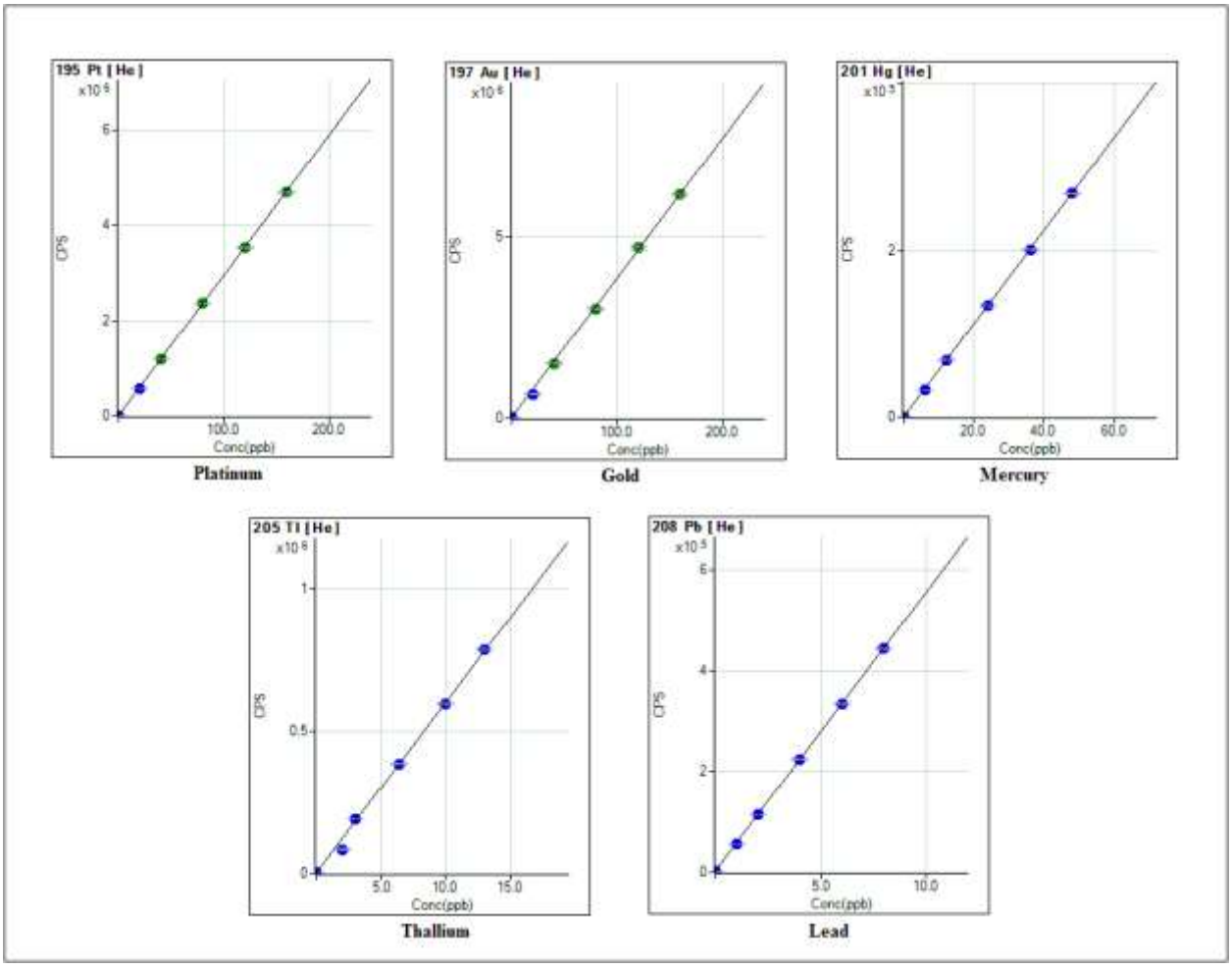

Fig 4 Calibration data for Pt, Au, Hg, TI, Pb

DISCUSSION

The present research work focused on simple and rugged ICP-MS method development and validation of 17 elemental impurities, i.e., Class 1 impurities Cd, Pb, As, Hg, Class 2A Co, V, Ni and Class 2B T1, Se, Ag, Au, Pd, Ir, Os, Rh, Ru, and Pt in propafenone hydrochloride drug substance. For the analysis of propafenone hydrochloride, drug substance sample digestion was done using nitric acid and sulfuric acid. Among the elemental impurities detected in propafenone hydrochloride drug substance samples, elemental impurities in class 1, impurities in class 2A & 2B, according to the elemental impurities' classification based on toxicity from ICH guidelines. Till date, no ICP-MS method was reported for the concurrent quantification of elemental impurities in propafenone hydrochloride drug substance. So, an attempt was made to develop simple, rapid ICP-MS method, and it was validated with precision, specificity, linearity, ruggedness, robustness, accuracy,

LOD and LOQ consecutively. Linearity obtained was with the acceptable prescribed limits respectively. The average recovery value was observed to be within the permissible limits. Estimated concentrations of these elements in drug substance samples were lower than the limits established by the chapter 232. [7]

For System suitability the correlation coefficient of calibration curve for each analyte was found to be > 0.99. Concentration of each analyte in bracketing standard was within the acceptance criteria (± 20% of actual concentration) from the obtained data it was concluded that system was suitable. The specificity studies demonstrated that the average (five times aspiration) of each calibration blank CPS and sample blank CPS for each analyte should not be more than 5% of 100% level standard solution CPS. From the observed data it was concluded that method was specific. The Limit of Quantification LOQ for each analyte should not be more than 30 % of the specification limit. LOQ for each analyte was found to be below 30% of the specification limit. The response of LOD solution for each element was found to be consistent. From the LOQ data the % RSD of class – 1, class – 2A & 2B elements response at LOQ level was found to be within 20%. Based on the observed data, it was concluded that the LOD and LOQ value for each elemental impurities reported values were observed to be at lowest possible level. For method precision the % RSD of the content of each elemental impurities in six samples were found to be less than 20 %. From the obtained data it was concluded that method was precise. The Correlation coefficient for each analyte was found to be >0.99. the residual sum of square, the intercept and the slope of the regression line were reported. Based on the linearity, precision and accuracy data, the range of the test method was from LOQ to 300 % of the target concentration was observed to be within the range. From the observed values, it was concluded that method was found to be linear. For method and intermediate precision % RSD of the content of each elemental impurities in six samples were found to be below 20 %. The cumulative % RSD for residue of class – 1, class – 2A & 2B elements (i.e. method and intermediate precision) for each analyte was found to be less than 25 %. From the observed data it was concluded that the method was precise and rugged. The mean % recovery for each analyte at each level was found to be within 70 % to 130 %. From the above data, it was concluded that method was accurate. For robustness the % RSD of the content of each elemental impurities in duplicate spiked samples were found to be below 20 %. From the data presented, it was observed that method was robust and precise. All the validation parameters such as system suitability, specificity, linearity, precision, LOD and LOQ, Accuracy and robustness compiled with the acceptance limits according to USP and ICH guidelines. In true sense, the daily maximum dose for propafenone hydrochloride and thus the risk is very little; therefore, the limits established considering this maximum daily dose may be elevated. [27-2]

CONCLUSION

A novel ICP-MS method was developed and validated according to current ICH and FDA guidelines to quantify 17 elemental impurities, class 1, Class 2A and 2B in propafenone hydrochloride. The proposed ICP-MS method has been evaluated to be precise, specific, linear, rugged, robust and accurate and proved convenient and effective for the quality control of propafenone hydrochloride. Thus, the present study demonstrates that ICP-MS has advantages over other conventional analytical methods for the determination of elemental impurities because of sensitivity, i.e., the lower limit of detection, for 17 elemental impurities in propafenone hydrochloride drug substance. Therefore, the method can easily be adopted for routine quantitative analysis of elemental impurities present as residual impurities in propafenone hydrochloride drug substance.

ABBREVIATIONS

ICP-MS: Inductively coupled plasma – Mass spectroscopy; RSD: Relative Standard Deviation; LOD:

Limit of Detection; LOQ: Limit of Quantitation; mL: Milliliter; ppm: Parts per million; ppb: Parts per billion; W: Watts; V: Volts; L/min: Liter/minute; rps: rotations per second; cps: Counts per second; mg: milligram; min: minutes; Hrs: Hours; °C: degree Celsius; µL: microliter; Sec: seconds; mm: millimeter; Std: Standard, USP: United States Pharmacopeia; ICH: International Council for Harmonization; NMT: Not more than; RSD: Relative Standard Deviation; FDA: Food and Drug Administration; HPLC: High Performance Liquid Chromatography; LC-MS: Liquid Chromatography Mass Spectrometry; UFLC-Ultra Fast Liquid Chromatography; PDE: Permitted daily exposure.

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تطوير واعتماد أسلوب بسيط وحساس لنظام ICP-MS لقياس الشوائب الأساسية في مادة هيدروكلوريد البروبافينون

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ملخص

الشوائب الأساسية هي مواد موجودة في المنتجات الدوائية، أو المخارج، أو التركيبات الدوائية. وقد تتكون بوجود محفزات وملوثات بيئية. يمكن اكتشاف الشوائب الأساسية عن طريق طريقة متطورة مثل القياس الطيفي للكتلة البلازمية المقترن بالحث (.(ICP-MSيعد ICP-MS طريقة متقدمة للكشف عن الشوائب الأساسية في المواد الدوائية. في هذه الدراسة تم إستخدام دواء بروبافينون هيدروكلوريد، وهو دواء مضاد لاضطراب النظم تابع للفئة 1 ج يستخدم لمنع عدم انتظام ضربات القلب فوق البطيني والبطين.تهدف هذه الدراسة إلى تطوير واعتماد طريقة التنظير الطيفي للكتلة البلازمية المقترنة بالتحريض لاكتشاف الملوثات الأساسية، أي الفئة 1 والأقراص المضغوطة والبطاقة الأساسية والشوائب من الفئة 2 ألف والزئبق والكيميائية والخامسة وشوائب الفئة 2 باء مثل الشوائب من الفئة 2 ألف، T1، Se، Ag، Au، Pd، Ir، Os، Rh، Ru، و .PTتم الكشف عن 17 شوائب أساسية في هيدروكلوريد البروبافينون، واستخدمت هذه الطريقة في التحليل المنتظم لعينة من 17 شوائب أساسية في هيدروكلوريد البروبافينون للاستخدام الصيدلاني. وحددت شروط الصك باستخدام قوة الترددات اللاسلكية البالغة 1550 واط، والغاز الإضافي 0.5 لتر/دقيقة، وتدفق الرذاذ البالغ 1.01 لتر/دقيقة للضغط على مضخة الرذاذ يبلغ 0.10 آر بي، ودرجة حرارة غرفة الرذاذ تبلغ درجتين مئويتين، وكانت الحالة هي هو، وكان معدل التدفق 4.3 ملليلتر/دقيقة، وكان معدل التمييز في الطاقة 3.0 فولت. وتتسم التقنية بحساسية وقد تحدد الشوائب الأساسية المستصوبة ضمن الحدود التنظيمية المسموح بها عند وجود عناصر إضافية. وقد تبين أن نهج ICP-MS المقترح دقيق ودقيق وخطي وصلد وقوي وملائم لمراقبة جودة مادة بروبافينون هيدروكلوريد الدواء. وكانت نتائج الخطية لكل شوائب 0،9990. وقد تم التحقق من صحة هذه الأساليب وفقا لمتطلبات USP والمبادئ التوجيهية للمجلس الدولي لتنسيق تكنولوجيا المعلومات والاتصالات. والنهج المقترح هو أداة ممتازة لمراقبة الجودة للتقييم الكمي المتزامن، والكشف عن الملوثات الأساسية عند مستويات منخفضة في هيدروكلوريد مادة العقاقير البروبافينون.

الكلمات الدالة: الشوائب الأساسية، ICP-MS، هيدروكلوريد البروبافينون، التحقق.

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The Impact of Online Education of Practical Courses on Pharmacy Students Practical and Communication Skills: Students' Perceptions

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ABSTRACT

The quarantine during the COVID-19 pandemic has forced universities to continue their education courses remotely, including practical courses. However, delivering practical laboratory courses was challenging, since all simulation laboratory courses lack real hands-on experience. The purpose of this study was to assess student's perception of the impact of online delivery of hands-onlaboratory courses, on pharmacy students' practical and communication skills. An anonymous Microsoft®Forms-based cross-sectional questionnaire was sent to potential participants at the University of Jordan Pharmacy School. Students' responses were analysed using SPSS® 23.0 software. A total of 274 online surveys were completed. About 69% of students preferred the hands-on laboratory courses and about 62% of students did not find online labs as effective as hands-on laboratory courses. About 73% of students think that online learning negatively affected their practical skills. Approximately 76% of students think that direct working in the lab improves their communication skills. Overall, Students prefer the traditional lab for practical course learning and think that learning online has negatively affected their practical and communication skills. This emphasises that pharmacy schools should consider the nature of practical courses when it comes to online educational methods inclusion into their curricula, to maximize the benefits delivered to students while matching students' needs and preferences.

Keywords: Pharmacy Education, Online, Laboratory, COVID-19.

INTRODUCTION

Practical knowledge is a cornerstone in pharmacy education. For instance, Pharmacy students must learn the analytical methods used in drug analysis. Practical skills are usually delivered by conducting experiments in laboratory courses, using glassware and equipment [1,2]. However, the COVID-19 outbreak, have highlighted alternative methods for practical skills teaching [3]. Among the most used alternative methods to deliver the practical laboratories by universities around the globe were the Video-based laboratories, in which students watch a demonstration video for the experiment, and virtual laboratories, in which students conduct experiment online in a virtual environment [4,5]. Distance teaching enables students to carry out experiments without safety concerns, like chemicals hazards, compared to laboratories. Moreover, online experiments are usually less stressful and take a shorter time [4]. All those benefits which are associated with online learning encouraged many higher education institutions to adopt the blended learning approach that implements the online learning technologies with the traditional in-class learning methods. Hence, providing students with the best learning opportunities [6].

However, during online laboratory sessions students are physically unable to touch the laboratories glassware

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and equipment. This lack of the real hands-on experience, may negatively affect students' practical as well as their communication skils[4,7]. In laboratory courses, students deal directly with their colleagues and instructors, share glassware, and many times have students to work in pairs or groups. All of which, will be missed or compromised during online education[8].

By the beginning of March 2020, the school of Pharmacy at the University of Jordan, laboratory sessions were cancelled and displaced by videos, shared online with students, showing demonstrations for the experimental work. The aforementioned videos were helpful to students to learn the basic concepts of the experiments; nevertheless, pharmacy students missed the real hands-on skills. The full resumption of hands-on laboratories was not started until the first semester of 2022.

These two years of intermittent online learning affected all pharmacy school students, especially the current third-year pharmacy students who have taken most of their first and second-year practical courses via online learning. Basic pharmacy students' practical skills are usually built during the first and second years. Hence, this must have led to difficulties faced by third-year students, after resuming hands-on laboratory works, in advanced courses, while lacking the hands-on experience from their previous years. Therefore, current third-year students might be the most affected by online learning during the pandemic.

Data is lacking regarding the consequences of online learning on pharmacy students practical and communication skills. Therefore, in the present research, we aim to gain insight into pharmacy student's perception of the impact of online learning, during the COVID-19 pandemic, on their practical and communication skills. This can help in making feedback recommendations for decisions makers in pharmacy school, to compensate for the missed hands-on skills in order to achieve the intended learning outcomes of the curriculum, either by offering compensatory courses or embedding the missed skills in other related courses. That will prevent the probability of graduating some students with inadequate laboratory skills that may have a negative impact on them when they enter the work market. Further, we aim to find out students' attitudes toward online learning. Online learning has shown some advantages that can be utilized, even after the end of the pandemic. For instance, video-based experiments can be a useful resource for students to prepare before the hands-on experiment. This can help them to follow safety measures according to the level of risks associated with the experiment. Moreover, online learning for practical courses can be a possible alternative to using expensive laboratory glassware and equipment, especially for low the income countries.

Experimental

Data collection

Data for this study were collected using an anonymous Microsoft®Forms based cross-sectional questionnaire. The data collection tool was developed by the authors based on the authors' experience and knowledge in the field and after intensive review of the literature [9–11].The questionnaire consists of four sections. The first section composed of items related to demographics and participants characteristics including gender, residence, nationality, whether secondary school belongs to public or private sector, GPA, academic level, and the program they are enrolled in. The second section covered items related to the effect of online sessions on students' practical skills, these items asked if not practicing experiments during the online learning period has affected students' practical skills and their abilities to use glass wares, handle reagent bottles and operate laboratory equipment. The third section asked questions about the effect of online learning stage on students' skills that are being developed during hands-on practical sessions including communication skills, abilities to active listening, self-confidence, patience and active engagement with colleagues and instructors. In the fourth section, students' preferences toward learning methodology were examined. Sections two, three and four

consisted together of 21 items on 5-point Likert scale ranging from 1 (strongly disagree) to 5 (strongly agree).

At the beginning of the questionnaire, a short and full description of the study scope and aims was added, in addition to that an informed consent statement to indicate participation agreement was required before the participant is allowed to answer the study questions.

Before the administration of the questionnaire, the data collection tool was assessed by expert in the field with long experience in teaching practical courses then a pilot study was conducted to test the data collection tool and 10 random responses were collected. Further evaluation of the tool was done using statistical confirmation of the tool validity and reliability. Cronbach's Alpha value of 0.702 asserts the internal consistency of the tool. Also, sample adequacy was confirmed factor analysis with Kaiser-Meyer-Olkin (KMO) value of 0.862 and a significant Bartlett's Test ($p<0.0001$).

Data collection took place from midDecember2021 till midMarch 2022. First and second year students were excluded. The tool was sent to potential participants via Microsoft Teams®, Facebook® and Emails. The questionnaire was randomly distributed to 308 students and 274 students completed with response rate of 88.9%.

To minimize social desirability bias, assurance was given to participants that the responses would be anonymized. Collected data was stored with the corresponding author and further analysis was done anonymously.

Statistical analysis

According to the registration department, the total number of students enrolled in 3rd, 4th, 5th and 6th year students in the School of Pharmacy at the University of Jordan is 950 - 1000 students, a sample size of 270- 278 participants was assumed to be sufficient as calculated via Raosoft® sample size calculator, using 95% confidence level and 5% margin of error [12].). Moreover, the total number of items in the questionnaire is 21 and applying the rule of the number of responses to item ratio ranges from 10:1[13], the collected responses were also sufficient.

Data analysis was conducted using SPSS® 23.0 (IBM, Armonk, NY) where data was encoded first then entered and analyzed. Responses were then presented as frequencies and percentages for categorical variables, and as means and standard deviations (or medians and inter-quartile ranges) for continuous variables. Comparisons between groups were performed using chi-square test. A *p*-value of < 0.05 was considered significant. All hypothesis testing was two-sided. For the purpose of comparisons, the 5-likert scale was shorten to 3-likert scale in which strongly agree and agree responses were merged and on the other side strongly disagree and disagree were merged. The two compared group were third year students (group 1) and fourth, fifth- and sixth-year students as group 2. This grouping was based on the fact that students from the third year were enrolled in the university during the COVID-19 pandemic lockdown and have no hands-on practical session's experience, while group 2 students have at least one-year experience with real face to face (F2F) practical session's experience (senior students). The study was approved Institutional Review Board (IRB) at the Deanship of Academic Research—The University of Jordan (IRB Ref. 9-2022). Besides, all methods were carried out following the national guidelines and conforming to the ethical standards of the *Declaration of Helsinki*. The questionnaire ensured the confidentiality and anonymity of study participants.

RESULTS

A total of 274 pharmacy students responded to this survey, of them 158 (57.7%) were third-year students and 116 (42.3%) were fourth, fifth- and sixth-year students (senior students). The majority 227 (82.8%) of the study respondent were females and Amman residents 224 (81.8%). Most of the respondents 157 (57.3%) academic performance was very good (See Table1).

Table 1: Students demographics and characteristics, N=274

		All (N=274)	Third Year Students (N=158)	Senior Students[a] (N=116)
		274 (100%)	158 (57.7)	116 (42.3)
Gender	Female	227 (82.8)	138 (87.3)	89 (76.7)
	Male	47 (17.2)	20 (12.7)	27 (23.3)
Residence	Amman	224 (81.8)	121 (76.6)	103 (88.8)
	Others	50 (18.2)	37 (2.6)	13 (11.2)
Nationality	local	230 (83.9)	134 (84.8)	96 (82.8)
	Others	44 (16.1)	24 (15.2)	20 (17.2)
Type of Secondary School	Governmental	142 (51.8)	90 (57)	52 (44.8)
	Private	132 (48.2)	68 (43)	64 (55.2)
Program	BSc of Pharmacy	175 (63.9)	86 (54.4)	89 (76.7)
	PharmD	99 (36.1)	72 (45.6)	27 (23.3)
Academic Level	Third year	158 (57.7)		
	Fourth year	67 (24.5)		
	Fifth year	43 (15.7)		
	Sixth year	6 (2.2)		
GPA	Excellent	77 (28.1)	48 (30.4)	29 (25)
	Very good	157 (57.3)	93 (58.9)	64 (55.2)
	Good	37 (13.5)	15 (9.5)	22 (19)
	Fair	3 (1.1)	2 (1.3)	1 (0.9)

[a]Fourth, fifth and sixth years students

As shown in table 2, The majority of third-year students agreed that they have faced difficulties working with laboratory glassware 62 (39.2%) and operating the laboratory equipment 74 (46.8%) after resuming laboratory courses, on the contrary, the majority of senior students disagreed with having such difficulties 55 (47.4%), 53 (45.7%) for glassware's and equipment respectively), the difference was significant between the third-year students and seniors' students' responses (P=0.001 and 0.002 for glassware's and equipment respectively). On the other hand, most students didn't find difficulties in handling reagent bottles after resuming hands-on laboratory courses137 (50.4%). Most students agreed that seeing (not handling) the lab glassware's and equipment during the online labs negatively affected their practical skills 200 (73%). And when students were asked if they think that the online labs didn't affect their practical skills most students disagreed 137 (50%).

Table 2: Effect of online delivery of practical sessions on students' practical skills

Statement	All N(%) (N=274)			Third Year Students N(%) (N=158)			Senior Students N(%) (N=116)			*p-value*[a]
	Agree	**Neutral**	**Disagree**	**Agree**	**Neutral**	**Disagree**	**Agree**	**Neutral**	**Disagree**	
I think that seeing (not handeling) the lab glassware's and equipment during the online labs negatively affected my practical skills.	200 (73)	42 (15.3)	32 (11.7)	121 (76.6)	21 (13.3)	16 (10.1)	79 (68.1)	21 (18.1)	16 (13.8)	0.295
After resuming real labs, I face difficulties working with laboratory glassware's (using a pipette for example).	94 (34.3)	84 (30.7)	96 (35)	62 (39.2)	55 (34.8)	41 (25.9)	32 (27.6)	29 (25)	55 (47.4)	0.001
After resuming real labs, I have difficulties in operating the laboratory equipment.	106 (38.7)	70 (25.5)	98 (35.8)	74 (46.8)	39 (24.7)	45 (28.5)	32 (27.6)	31 (26.7)	53 (45.7)	0.002
After resuming real labs, I have difficulties in handling the laboratory reagent bottles.	51 (18.6)	85 (31)	138 (50.4)	34 (21.5)	52 (32.9)	72 (45.6)	17 (14.7)	33 (28.4)	66 (56.9)	0.148
I think that learning online didn't affect my practical skills in the lab.	83 (30.3)	54 (19.7)	137 (50)	44 (27.8)	29 (18.4)	85 (53.8)	39 (33.6)	25 (21.6)	52 (44.8)	0.340

[a] *Pearson Chi-square*

Table 3 illustrates the effect of online delivery of practical sessions on students' perception of their communications skills. Students agreed that online learning decreased their active listening skills 166 (60.6%). Regarding the self-confidence skill, there was a significant difference between third year and senior's students' responses, the majority of third-year students agreed 81 (51.3%) that online learning decreases their self-confidence, however, the majority of seniors 41 (35.3%) disagreed. Students disagreed that they tend to avoid eye contact after resuming hands-on laboratory courses 148 (54%), but the percentage of students who disagreed was higher in the senior year group 72 (62.1%) than the third-year group 76 (48.1%). Most students agreed that they feel impatient during hands-on laboratory courses sessions 137 (50%) and agreed that it was easier to communicate with the lab instructor during the hands-on laboratory courses compared to the online lab 188 (68.6%). Most students agreed that working as a group in a hands-on labs has improved their communication skills 209 (76.3%) but there was a significant difference between the students' groups (p=0.019).

Table 3: Effect of online delivery of practical sessions on students' communication skills

Statement	All N(%) (N=274)			Third Year Students N(%) (N=158)			Senior Students N(%) (N=116)		
	Agree	Neutral	Disagree	Agree	Neutral	Disagree	Agree	Neutral	Disagree
I think that working in pairs/a group with my colleagues in real lab, improved my communication skills.	209 (76.3)	34 (12.4)	31 (11.3)	116 (73.4)	17 (10.8)	25 (15.8)	93 (80.2)	17 (14.7)	6 (5.2)
I think that online learning decreased my active listening ability.	166 (60.6)	59 (21.5)	49 (17.9)	97 (61.4)	31 (19.6)	30 (19)	69 (59.5)	28 (24.1)	19 (16.4)
I think that online learning decreased my self-confidence.	121 (44.2)	69 (25.2)	84 (30.7)	81 (51.3)	34 (21.5)	43 (27.2)	40 (34.5)	35 (30.2)	41 (35.3)
After resuming real labs, When the lab instructor gives me instructions, I affirm that I understand, even if I don't entirely understand.	109 (39.8)	81 (29.6)	84 (30.7)	68 (43)	46 (29.1)	44 (27.8)	41 (35.3)	35 (30.2)	40 (34.5)
After resuming direct learning, I found myself avoiding eye to eye contact with my colleagues and instructors.	55 (20.1)	71 (25.9)	148 (54)	37 (23.4)	45 (28.5)	76 (48.1)	18 (15.5)	26 (22.4)	72 (62.1)
I feel impatient during the real lab sessions (for example: when you have to wait for your turn for using a certain device or equipment).	137 (50)	77 (28.1)	60 (21.9)	83 (52.5)	44 (27.8)	31 (19.6)	54 (46.6)	33 (28.4)	29 (25)
During the real lab sessions, I found it easier to ask for clarification when my instructor says something I'm not sure about compared to the online lab.	188 (68.6)	63 (23)	23 (8.4)	112 (70.9)	33 (20.9)	13 (8.2)	76 (65.5)	30 (25.9)	10 (8.6)

[a] *Pearson Chi-square*

Table 4 demonstrates students' preferences regarding F2F and online learning methods for practical courses. More than two thirds of students felt more motivated after resuming hands-on laboratory courses199 (72.6%), with a significant difference between a third year and seniors' student's responses(p=0.003). And when students were asked if they preferred online lab sessions, most of students disagreed 189 (69%). Students disagreed that online lab delivery enabled them to continue their education like the direct lab 132 (48.2%) with a significant difference between third year and senior's students' responses (p=0.003). Students disagreed that online labs enabled them to understand the experiment without safety concerns compared to hands-on experiments 137 (50%) and the difference was significant between third year and senior's students' responses(p=0.016). Most students 132 (48.2%) felt that direct lab assessment is more stressful than online assessment. And disagreed that they feel that hands-on lab is time-consuming compared to the online lab 125 (45.6%). In addition, most students think that direct lab allows for a higher chance of COVID-19 transmission 121 (44.2%), but there was a significant difference between third and seniors' year student's responses (p=0.001).

Table 4: Effect of online delivery of practical sessions on students' preferences

Statement	All N(%) (N=274)			Third Year Students N(%) (N=158)			Senior Students N(%) (N=116)			*p-value*[a]
	Agree	**Neutral**	**Disagree**	**Agree**	**Neutral**	**Disagree**	**Agree**	**Neutral**	**Disagree**	
I feel more motivated after resuming the direct face to face laboratory work.	199 (72.6)	56 (20.4)	19 (6.9)	126 (79.7)	21 (13.3)	11 (7)	73 (62.9)	35 (30.2)	8 (6.9)	0.003
I prefer online labs sessions compared to real lab sessions.	40 (14.6)	45 (16.4)	189 (69)	20 (12.7)	26 (16.5)	112 (70.9)	20 (17.2)	19 (16.4)	77 (66.4)	0.560
I feel that direct lab assessment is more stressful than online assessment.	132 (48.2)	69 (25.2)	73 (26.6)	71 (44.9)	40 (25.3)	47 (27.7)	61 (52.6)	29 (25)	26 (22.4)	0.339
I think that online delivery of lab content enables students to continue their education similar to the direct lab.	68 (24.8)	74 (27)	132 (48.2)	47 (29.7)	31 (19.6)	80 (50.6)	21 (18.1)	43 (37.1)	52 (44.8)	0.003
I feel that online labs allow me to understand the real experiments without safety concerns compared to the real lab.	81 (29.6)	56 (20.4)	137 (50)	36 (22.8)	36 (22.8)	86 (54.4)	45 (38.8)	20 (17.2)	51 (44)	0.016
I think that the real lab is time consuming compared to the online lab.	88 (32.1)	61 (22.3)	125 (45.6)	47 (29.7)	37 (23.4)	74 (46.8)	41 (35.3)	24 (20.7)	51 (44)	0.607

Statement	All N(%) (N=274)			Third Year Students N(%) (N=158)			Senior Students N(%) (N=116)			*p-value*[a]
	Agree	**Neutral**	**Disagree**	**Agree**	**Neutral**	**Disagree**	**Agree**	**Neutral**	**Disagree**	
I think that direct working in the lab allows for a higher chance for COVID-19 transmission.	121 (44.2)	85 (31)	68 (24.8)	58 (36.7)	49 (31)	51 (32.3)	63 (54.3)	36 (31)	17 (14.7)	0.001
I think that I need extra face to face classes to compensate for what I missed during online lab learning.	127 (46.3)	75 (27.4)	72 (26.3)	87 (55.1)	38 (24.1)	33 (20.9)	40 (34.5)	37 (31.9)	39 (33.6)	0.003
I think that online lab experience is effective as real lab experience.	49 (17.9)	55 (20.1)	170 (62)	22 (13.9)	25 (15.8)	111 (70.3)	27 (23.3)	30 (25.9)	59 (50.9)	0.005

[a] *Pearson Chi-square*

When students were asked if they think that they need extra F2F classes to compensate for what they missed during online labs, more than half of third-year students 87 (55.1%) agreed, while only 40 (34.5%) of seniors agreed, the difference was significant (p=0.003). Most students didn't agree that the online lab was effective as a hands-on lab 170 (62%), and the difference between third year and senior year students' responses was significant (p=0.005).

DISCUSSION

Overall, students preferred hands-on laboratory sessions and did not find online labs as effective as F2F labs in increasing their practical and communication skills. Students didn't find value in seeing laboratory glassware and equipment without the hands-on experience and think that their practical skills have been negatively affected by online learning. Working together in hands-on lab sessions, still be considered valuable by students to improve their communication skills. Students think that their ability to listen actively and their self-confidence have been decreased by online learning. Further, Students find it easier to communicate with collogues and instructors during hands-on F2F labs compared to the online labs. Interestingly, students didn't find hands-on F2F lab sessions time consuming, even though hands-on lab sessions take a longer time compared to online lab session. From another view, students think that hands-on F2F labs assessment is more stressful than the online labs. Moreover, students think that direct working in a hands-on F2F lab allows for a higher chance for COVID- 19 transmittance.

When students' responses were stratified according to school academic year, significant differences were found

when comparing third-year students to senior students (fourth, fifth and sixth years) students, with prior exposure to hands-on F2F practical sessions before the COVID-19 pandemic. Third-year students have faced difficulties using laboratory glassware and equipment, after resuming hands-on F2F labs, more than the senior students. This may be explained by the effect of previous laboratory hands-on experience which senior students have been exposed to during their early academic years. Third-year students lack this experience due to the lockdown during the COVID-19 quarantine. Therefore, third-year students think they need extra F2F classes to compensate for the missed skills and were more motivated after resuming hands-on F2F labs.

Senior students value the beneficial effects of working as groups, during hands-on F2F lab on their communication skills more than the third-year students. In addition, senior students didn't think that their self-confidence was negatively affected by online learning. On the contrary, third-year students do think that their self-confidence was negatively affected. Interestingly**,** senior students believe that working in a hands-on F2F lab allows for a higher chance for COVID-19 transmittance, more than third-year students. This again may be explained by the earlier hands-on F2F laboratory experience senior students have, which makes them more familiar with the nature of interactions during scientific F2F labs which could lead to diseases transmittance. Those differences between the third year and senior students' responses, agree with the results of studies conducted by Hamilton *et al* and Survey *et al* which show that students' attitudes and preferences can change as they advance in their academic year. Accordingly, caution must be taken when designing and reviewing curricula to take into consideration that students' academic year affects their preferences[11,14].

Previous studies have shown that online learning is effective for pharmacy education in the short term. Further, the use of online learning in pharmacy education has several benefits; it renders more convenience and time flexibility when compared to traditional learning. Moreover, the use of online learning in practical courses gets over safety and health pitfalls associated with hands-on laboratory courses. In addition, many medical schools have started to utilize online labs as a cost-effective alternative to hands-on F2F labs, due to the high cost of the lab's equipment and budget shortage. Nevertheless, the majority of studies have shown that pharmacy students preferred the blended learning approach [4,6,11,15,16].

Students in this study did not prefer the use of online learning in practical courses. The results of this study agree with a previous study conducted by Survey *et al* where students in the biology lab preferred hands-on F2F lab sections to the online lab [14] and agree with results of Ali *et al* previous study where pharmacy students preferred the hands-on F2F labs for practical courses[17]. Another study also have shown that medical students preferred F2F microbiology labs compared to online laboratory[18] In the contrary, a recent study has shown that pharmacy students at a University in Spain were satisfied with online learning of chemistry laboratory courses, implemented during the COVID-19 pandemic, where students performance was improved by online learning compared to the traditional laboratory methods[19]Another study from Thailand has shown that pharmacy students were satisfied with the learning outcomes of online learning of medicinal chemistry laboratorycourses[20]

However, Students' preferences should be taken alongside the best teaching practices, which may not be always in one line. Therefore, the use of a blended learning approach can be a suitable choice for practical courses to optimize the benefits students can gain from both the traditional learning and online learning method. Hence, The University of Jordan pharmacy school has adopted the blended learning approach for practical courses, where the theoretical component of the labs is given online to students while keeping the practical part in the F2F labs.

However, this study has some limitations; the participants in the survey were University of Jordan pharmacy students only. Therefore, generalizing those results to other students' communities must be done

carefully. Moreover, students were surveyed about online labs in general without specifying a certain lab; during pharmacy study, students encounter different labs of different nature and different degrees of hands-on skills required for students to have. Consequently, some labs can be delivered online without major effects on students' practical skills, while delivering other labs online can truly affect students' practical skills.

CONCLUSIONS

Overall, students did not prefer online learning for practical courses delivery and thought that online learning has a negative impact on their practical and communication skills. Therefore, consideration must be done to continue the traditional F2F learning that is merged with an online component like recorded experiments in the form of blended learning for practical courses, to satisfy students' needs and preferences while maintaining a high quality learning outcomes The negative impact of online learning was seen in third-year students more than senior students; therefore, pharmacy schools should consider the students' academic year while incorporating online courses into their educational curriculum.

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تأثير التعلم عن بعد على إيصال المواد العملية لطلاب كلية الصيدلة:توجهات الطلاب

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ملخص

أجبر الحجر الصحي خلال جائحة كورونا الجامعات إلى متابعة برامجها التعليمية عن بعد, بما فيها المختبرات العملية. لكن إيصال المختبرات العملية واجه العديد من التحديات نظرًالأن جميع بدائل التعلم المباشر داخل المختبرات تفتقد الخبرة العملية الحقيقية. لهذا كان الهدف من هذه الدراسة هو تقييم مدى تأثيرالتعلم الإلكتروني للمختبرات العملية على المهارات العملية ومهارات الإتصال لدى طلبة كلية الصيدلة في الجامعة الأردنية من وجهة نظر الطلاب. لتحقيق هذا الهدف تم إرسال استبيان إلكتروني لطلبة كلية الصيدلة في الجامعة الأردنيةوتحليل نتائج هذا الإستبيان عبر برمجية التحليل الإحصائي SPSS.أكمل 274 طالب الإستبيان, حوالي 69% من الطلاب فضل المختبر التقليدي وما نسبته 62% من الطلاب لم يجدو أن المختبرات المعطاة عبر الإنترنت بفعالية المختبر المختبرات التقليدية. حوالي 73% من الطلاب يعتقد أن تعلم المختبرات عبر الإنترنت أثر سلبًا على مهاراتهم العملية وحوالي 76% من الطلاب يعتقد أن المختبرات التقليدية تحسن من مهارات التواصل لديهم. بشكل عام يفضل الطلاب المختبرات المعطاة عن طريق الطرق التقليدية ويعتقد الطلاب أن المختبرات المعطاة عبر الإنترنت تئثر بشكل سلبي على مهاراتهم العملية والتواصلية.يؤكدهذاعلى أن مدارس الصيدلة يجب أن تأخذ في الإعتبار طبيعة المختبرات العملية عندمايتعلق الأمربإدراج الأساليب التعليمية عبرالإنترنت في مناهجها ،لتعظيم الفوائد المقدمة للطلاب مع مطابقة احتياجات الطلاب وتفضيلاتهم.

الكلمات الدالة: الصيدلة، التعليم، الإنترنت، المختبرات.

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Evaluation of the Effect of Dapagliflozin on CRP Levels in Type 2 Diabetes Patients

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is an increasingly prevalent chronic disease that associates with an increased risk of micro-and macrovascular complications. There is persuasive evidence that dapagliflozin may reduce chronic inflammation besides its glucose-lowering effect, which in term prevents the development of the disease and its complications. Therefore, this study aims to evaluate the effects of dapagliflozin on the inflammatory marker C-reactive protein (CRP) levels in T2DM patients. Patients with T2DM were randomly assigned into two groups, group 1 (n=52) receiving a daily dose of dapagliflozin as an add-on therapy with oral antihyperglycemic agents, and group 2 (control, n=60) who received oral antihyperglycemic agents (Metformin, Sulfonylureas, Thiazolidinediones, and Gliptins). After six months, our results showed a significant change in CRP levels from baseline after receiving dapagliflozin compared to the control. Although the reduction level of CRP was statically significant with both 5 mg and 10 mg doses, it was higher with the latter one. In addition, the reduction in CRP levels was statistically significant in both controlled and uncontrolled, but more important in uncontrolled disease. An insignificant positive correlation was seen between HbA1c and CRP on admission (r: 0.21, p: 0.1) and during the follow-up period, at 3 months (r: 0.10, p: 0.4) and 6 months (r: 0.08, p: 0.5). Our study showed that dapagliflozin has a beneficial effect on inflammation by reducing CRP levels CRP in patients with T2DM.

Keywords: Type 2 diabetes mellitus (T2DM), Dapagliflozin, CRP, inflammation.

INTRODUCTION

Diabetes mellitus (DM) encompasses metabolic disorders that are characterized by hyperglycemia resulting from relative or absolute impairment of insulin secretion with varying degrees of insulin resistance. According to etiology and clinical presentation, DM is classified into three types: type 1 (T1DM), type 2 (T2DM), and gestational (GDM)[1]. The prevalence of DM has increased dramatically over the past four decades. It is considered a worldwide epidemic with increasing obesity and lifestyle alterations[2].

Type 2 diabetes mellitus (T2DM) is a progressive disease that can be divided into four stages: defect in β cell glucose-stimulated insulin secretion, peripheral insulin resistance, β cell compensations, and β cell loss[3]. T2DM has been correlated with increased levels of inflammatory markers, including interleukin (IL-6), tumor necrosis factor-alpha (TNF-α), plasminogen activator inhibitor 1 (PAI-1), C-reactive protein (CRP), and chemokines. Furthermore, factors that are released from adipose tissue (adipokines) stimulate inflammatory activity, which correlates with insulin resistance[4&5]. Increased CRP blood level is considered a sign of systemic inflammation. It is synthesized and secreted primarily in hepatocytes and

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regulated by IL-6, IL-1, and TNF-α [6].

Early initiation of diabetes treatment is associated with improved glycemic management and decreased long-term complications[7]. Sodium-glucose co -transporter-2 inhibitors (SGLT2i) are new drugs that promote the renal excretion of glucose and thereby lower high-risk blood glucose levels in patients with T2DM[8]. Currently, there are three FDA-approved SGLT2 selective inhibitors for the treatment of T2DM: canagliflozin, dapagliflozin, and empagliflozin[9]. The anti-inflammatory properties of dapagliflozin may possess therapeutic benefits beyond their glucose-lowering activity. Several mechanisms explain the anti-inflammatory effect of dapagliflozin including a reduction in adipose tissue inflammation, weight loss, mild increase in ketone bodies, and attenuation of oxidative stress[10]. The objective of this study was to investigate changes in CRP levels during treatment with dapagliflozin in T2DM patients.

MATERIALS AND METHODS

This is a randomized controlled trial of a group of T2DM patients attending the department of endocrinology at Tishreen University Hospital in Lattakia-Syria from September 2020 to May 2022.

We included male and female patients from different age groups who were diagnosed with T2DM.

On the other hand, we excluded patients with one of the following: chronic inflammatory disease, pathologic obesity, changed or discontinued drugs that affected CRP values during follow-up, acute inflammation when specimen samples were collected and estimated glomerular filtration rate (e GFR) ≤ 30 ml/min/1.73 m².

A questionnaire was designed to record patients' information such as age, weight, height, Body mass index (BMI), current and previous diseases, used medications, and smoking.

The first group included 52 T2DM, who received dapagliflozin as an add-on therapy to oral antihyperglycemic drugs (Metformin, Sulfonylureas, Thiazolidinediones, and Gliptins). The second group (control) included 60 T2DM, who were on oral antihyperglycemic drugs (Metformin, Sulfonylureas, Thiazolidinediones, and Gliptins). Patients in group I were on a daily dose of either 5 mg (27 cases) or 10 mg (25 cases) of dapagliflozin. Patients were on the same medication regimen during the follow-up period without adding any drug that can affect CRP values such as oral contraceptives or statins.

Body mass index (BMI) was calculated as (weight/height^2) (kg/m^2) and categorized as normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2), and obesity (≥30 kg/m^2). Patients were divided according to the 10-year atherosclerotic cardiovascular disease (ASCVD) risk score developed by the American College of Cardiology/American Heart Association (ACC/AHA) into a low-risk group if ASCVD score was ≤7.5% and a high-risk group if ASCVD score was >7.5%. Glycated hemoglobin (HbA1c) was considered as a marker of good glycemic control if HbA1c was <7% and poor glycemic control if HbA1c was >7%. CRP levels were measured in all patients at baseline, after 3 months, and after 6 months.

Biochemical evaluation:

Venous blood samples were taken in the morning after fasting for at least 8 hours overnight.

Laboratory assessments included:

1. C reactive protein (CRP) levels were measured by turbidometric method using an automated analyzer (BS-380, Mindary) normal range: < 5 mg/L.
2. Fasting plasma glucose levels were measured by colorimetric method using an automated analyzer (BS-380, Mindary) normal range: 70-110 mg/dL
3. Glycated hemoglobin HbA1c was measured by fluorescent immunoassay technology (Diabetes >6.5%) by an automated analyzer (Finecare, Wondfo).

Ethical approval: All procedures were approved by the Institutional Review Board of Tishreen University. The decision involved Ethical Approval (Decision Number: 2874 in September 2020). Informed verbal consent was taken from each participant in the study.

Statistical Analysis:

Statistical analysis was performed using IBM SPSS version20. Basic Descriptive statistics included means, standard deviations (SD), median, frequency, and percentages. For relationships and comparisons between two groups, the chi-square test or Fisher exact test was performed. An Independent t-student test was used to compare 2 independent groups. The Friedman test was used to detect differences between groups when the dependent variable being measured was ordinal. All tests were considered significant at a 5% type I error rate (p<0.05), β: 20%, and power of the study: 80%.

RESULTS

Study's group included 112 patients (51 male, 61 female) with T2DM. Age ranged from 39 to 70 years, with a mean age of 54.8±7.7 years, BMI ranged from 18.73 to 35 kg/m² with a mean value of 28.1±3.6 kg/m², and HbA1c ranged from 4.20 to 11.20 mg/dL with a mean value of 7.50±1.3 mg/dL.

The baseline characteristics of patients are shown in Table (1). No significant differences were found between groups in terms of age, gender, BMI, smoking, comorbidities, and drugs (p>0.05) except for using statins, which was more frequent in group II (45% versus 23.1%, p: 0.01). In group I, the mean age was 56.15±7.9 years, and the most frequent age group was 50-59 years (40.4%), followed by ≥ 60 years (34.6%) and 40-49 years (25%). Males represented 42.3% and females represented 57.7% of the patients. Mean value of BMI was 28.21±3.5 kg/m². Overweight patients represented the most frequent group (46.2%), followed by obesity (34.6%) and normal weight (19.2%). Hypertension was present in 40 cases (76.9%) and the mean duration of the disease was 4.08±3.74 years. Regarding frequencies of antidiabetic agents, 94.2% of the patients were on metformin, 50% were on sulfonylurea, 23.1% were on dipeptidyl peptidase inhibitors, and 1.9% were on thiazolidinediones. Patients were classified according to ASCVD score into low-risk (31 cases: 59.6%) or high-risk (21 cases: 40.4%). In group II, the mean age was 53.73±7.5 years, and the most frequent age group was 50-59 years (48.3%), followed by 40-49 years (28.3%), and ≥ 60 years (23.3%). Males represented 48.3% and females represented 51.7% of the patients. Mean value of BMI was 28.12±3.8 kg/m². Overweight patients represented the most frequent group (55%), followed by obesity (28.3%) and normal weight (16.7%). Hypertension was present in 36 cases (60%) and the mean duration of the disease was 3.5±2.49 years. All patients were on metformin, 46.7% were on sulfonylurea, 23.3% were on dipeptidyl peptidase inhibitors, and 1.7% were on thiazolidinediones. Patients were divided into low-risk in 37 cases (61.7%) and high-risk in 23 cases (38.3%).

Table 1. Comparison of demographic characteristics of the study groups.

Variables	Group I	Group II	*P*-value
	Dapagliflozin (n=52)	Control (n=60)	
Age (years)	56.15±7.9	53.73±7.5	0.1
Age group (years)			
40-49	13(25%)	17(28.3%)	0.4
50-59	21(40.4%)	29(48.3%)	
≥ 60	18(34.6%)	14(23.3%)	
Sex			
Male	22(42.3%)	29(48.3%)	0.5
Female	30(57.7%)	31(51.7%)	

Variables	Group I	Group II	*P*-value
	Dapagliflozin (n=52)	**Control (n=60)**	
BMI (kg/m2)	28.21±3.5	28.12±3.8	0.9
BMI group			
Normal weight	10(19.2%)	10(16.7%)	0.3
Overweight	24(46.2%)	33(55%)	
Obesity	18(34.6%)	17(28.3%)	
Smoking	26(50%)	34(56.7%)	0.4
Comorbidities			
• Hypertension	40(76.9%)	36(60%)	0.05
Duration (year)	4.08±3.74	3.5±2.49	0.08
• Coronary artery disease (CAD)	5(9.6%)	5(8.3%)	0.8
Oral antihypertensive drugs			
ACE inhibitors	12(23.1%)	10(16.7%)	0.3
Angiotensin receptor blockers (ARBs)	16(30.8%)	14(23.35)	0.3
Beta-blocker	14(26.9%)	9(15%)	0.1
Calcium channel blockers	10(19.2%)	12(20%)	0.9
Diuretics	6(11.5%)	6(10%)	0.7
Others			
Aspirin	6(11.5%)	8(13.3%)	0.7
Statins	12(23.1%)	27(45%)	0.01
Oral hypoglycemic drug			
Metformin	49(94.2%)	60(100%)	0.1
Sulfonylurea	26(50%)	28(46.7%)	0.5
Thiazolidinediones	1(1.9%)	1(1.7%)	0.9
Dipeptidyl peptidase inhibitors	12(23.1%)	14(23.3%)	0.3

The data was analyzed using Chi-Square test or Fisher exact test, $^{*}p$-value ≤ 0.05.

As shown in table (2), no significant difference was found in group I depending on the dose (5 mg or 10 mg) regarding age, gender, BMI, presence of comorbidities, duration, drugs, and the degree of diabetes control ($p>0.05$). Patients on dapagliflozin (5 mg) group were divided into low-risk in 15 cases (55.6%) and high-risk in 12 cases (44.4%), whereas patients on dapagliflozin (10 mg) group were assigned into low-risk in 16 cases (64%) and high-risk in 9 cases (36%), without significant difference between two groups ($p>0.05$).

Table 2. Comparison of demographic characteristics of dapagliflozin group based on dapagliflozin dose.

Variables	Group I		*P*-value
	Dapagliflozin 5 mg (n=27)	Dapagliflozin 10 mg (n=25)	
Age (years)	57.18±7.8	55.04±8	0.3
Sex			
Male	13(48.1%)	9(36%)	
Female	14(51.9%)	16(64%)	0.3
BMI (kg/m2)	28.10±3.3	28.27±2.2	0.5
Smoking	13(48.1%)	13(52%)	0.7
Comorbidities			
• Hypertension	22(81.5%)	18(72%)	0.4
Duration(year)	4.3±4.25	3.7±3.18	0.3
• Coronary artery disease(CAD)	4(14.8%)	1(4%)	0.1
Oral antihypertensive drugs			
ACE inhibitors	8(29.6%)	4(16%)	0.2
Angiotensin receptor blockers(ARBs)	8(29.6%)	8(325)	0.8
Beta -blocker	8(29.6%)	6(24%)	0.6
Calcium channel blockers	6(22.2%)	4(16%)	0.5
Diuretics	2(7.4%)	4(16%)	0.3
Others			
Aspirin	3(11.1%)	3(12%)	0.9
Statins	5(18.5%)	7(28%)	0.4
Oral hypoglycemic drug			
Metformin	26(96.3%)	23(92%)	0.2
Sulfonylurea	15(55.6%)	11(44%)	0.4
Thiazolidinediones		1(4%)	0.2
Dipeptidyl peptidase inhibitors	8(29.6%)	4(16%)	0.2
Duration of treatment T2DM(years)	4.40±3.01	3.93±2.2	0.5
HbA1c(mg/dl)	7.52±1.2	7.47±1.06	0.8

The data was analyzed using Chi-Square test or Fisher exact test, $^{*}p$-value ≤ 0.05.

As shown in table (3), the mean period between the treatment of T2DM and the study enrolment was 4.18±2.6 years in group I versus 3.57±3.2 years in group II, *p*: 0.2. Patients were divided into three groups according to treatment of the disease; <3, 3 – 7 and ≥7. The vast majority of diabetes cases fall in the category 3-7 in group I (63.5%) and group II (46.7%), without significant difference (*p*: 0.1). Mean HbA1c was 7.50±1.1 mg/dl in group I vs. 7.16±1.2 mg/dl in group II, *P*: 0.1. 31 patients (59.6%) exhibited relatively poor glycemic control at baseline vs. 32 patients (53.3%) in group II.

Table 3. Baseline characteristics of T2DM treatment and HbA1c of study's groups.

Variables	Group I	Group II	*P*-value
	Dapagliflozin (n=52)	Control (n=60)	
Duration of treatment T2DM (years)	4.18±2.6	3.57±3.2	0.2
<3	11(21.2%)	21(35%)	0.1
3 – 7	33(63.5%)	28(46.7%)	
≥7	8(15.4%)	11(18.3%)	
HbA1c (mg/dl)	7.50±1.1	7.16±1.2	0.1
Controlled	21(40.4%)	28(46.7%)	0.5
Uncontrolled	31(59.6%)	32(53.3%)	

The data was analyzed using Chi-Square test or Fisher exact test, $^{*}p$-value ≤ 0.05, HbA1c: hemoglobin glycosylated A1C.

As illustrated in Table 4, our results indicate that effect of dapagliflozin on lowering CRP levels is treatment duration dependent. Mean value of CRP was 4.82±4.1 mg/l at baseline and decreased to 2.23±2.2 mg/l after 6 months (*p*: 0.0001), whereas in control group there was no statistically significant difference in CRP-values during the follow-up duration (*p*: 0.08). Dapagliflozin's effect on CRP is dose-dependent. During follow-up, there was a significant decrease in CRP levels in patients who received a daily dose of 5 mg (2±1.8 mg/l at the end of 6 months vs. 4.26±2.9 mg/l at baseline, *p*: 0.0001), and in patients who received a daily dose of 10 mg (2.44±2.7 mg/l at the end of 6 months vs. 5.35±5.1 mg/l at baseline, *p*: 0.0001). However, the level of decrease was higher in patients who were on a daily dose of 10 mg. Additionally, CRP levels were decreased more significantly after 6 months when HbA1c levels were higher at baseline; 2.14±1.8 mg/l at the end of 6 months vs. 4.04±3.5 mg/l at baseline, *p*: 0.0001 in controlled group, and 2.29±2.5 mg/l at the end of 6 months vs. 5.35±4.5 mg/l at baseline, *p*: 0.001 in uncontrolled group. But the level of decrease was higher in patients with higher HbA1c. When CRP levels were higher at baseline, the decrease in CRP levels was higher after 6 months. In comparison to controlled patients, baseline CRP levels were higher in uncontrolled patients.

Table 4. Comparison of measurements between baseline and six-month treatment

Variables	CRP			
	Baseline	3 months	6 months	*P*-value
Dapagliflozin	4.82±4.1	3.21±3.2	2.23±2.2	**0.0001***
Control	3.74±2.5	3.63±2.5	3.49±2.6	0.08
P-value	0.07	0.4	0.006	
Dapagliflozin				
Controlled	4.04±3.5	2.98±2.3	2.14±1.8	**0.0001***
Uncontrolled	5.35±4.5	3.36±3.7	2.29±2.5	**0.001***
P-value	0.2	0.6	0.8	
Dapagliflozin				
5 mg	4.26±2.9	2.71±1.9	2±1.8	**0.0001***
10 mg	5.35±5.1	4.09±3.18	2.44±2.7	**0.0001***
P-value	0.3	0.2	0.4	

The data was analyzed using Friedman test and Independent T Student, * *p*-value ≤ 0.05.

A relationship between HbA1c and CRP was analysed, and we concluded that with low levels of HbA1c, there was an insignificant decrease in CRP at baseline, 3 months, and 6 months of therapy (r: 0.21, *p*: 0.1), (r:0.10, *p*:0.4), and (r: 0.08, *p*: 0.5).

DISCUSSION

The principal clinical concern with T2DM subjects is the potential development of clinically significant complications and associated morbidity and mortality. Therefore, it is crucial that research should focus on the prevention as well as the treatment of the disease.

The result of the current study revealed that dapagliflozin performed a significant reduction in CRP levels when compared to the control ($p<0.05$). The rate of reduction was higher in patients who received a daily dose of 10 mg more than those who took 5 mg, and in uncontrolled DM. Low levels of HbA1c were associated with an insignificant decrease in inflammation degree, which was represented by decreased CRP levels. These changes may be explained by the following effects of dapagliflozin. Firstly, the drug reduces oxidative stress[10], fibrosis[11], and sympathetic overdrive[12]. Secondly, it stimulates anti-inflammatory macrophages and anti-inflammatory cytokines (IL-10)[13&14]. Finally, it reduces renal pro-inflammatory cytokines (TNFα and IL-6), fibrosis, and apoptosis[15&16]. In addition, there were no confounding factors that may affect the CRP values during follow-up. The rate of using statins was higher in the control than the intervention group, which might explain the low baseline values of CRP in control group. Various studies have provided definitive evidence for the favorable effects of dapagliflozin in decreasing CRP levels. Okamoto et al (2016) conducted a study on 27 obese patients with T2DM who were treated with dapagliflozin 5 mg/day for 12 weeks. Their study showed a significant reduction in CRP levels (ng/ml); 1960±1607 vs. 2814±2410, $p<0.01$[17].

Moreover, a study conducted on T2DM mice revealed that a daily dose of dapagliflozin (1.5 mg/kg) reduced CRP levels significantly ($p<0.001$)[18].

Xue et al (2021) demonstrated in a study carried out on 70 patients with T2DM and ST-segment elevation myocardial infarction (35 cases, 35 controls) a significant decrease in IL-6, TNF-α, and CRP levels in patients who were treated with dapagliflozin 5 mg/day at the first week, followed by 10 mg/day compared to the control group, *p*: 0.001[19].

Alhwiesh et al (2022) showed in a study conducted on 50 T2DM patients in Saudi Arabia significantly lower values of CRP in patients receiving a daily dose of 10 mg of dapagliflozin for 6 months[20].

In contrast to our study, Zaihordin et al (2019) demonstrated in a study conducted in Malaysia on 81 T2DM patients with ischemic heart disease (dapagliflozin: control, 40:41) a significant increase in CRP levels: 6.03 vs. 1.93 at baseline, *p*:0.009[21]. Most of these patients were in a high-risk category, and they were followed up for just 12 weeks, dapagliflozin's anti-inflammatory effect may take a longer time to manifest clearly and statistically significant[21].

In summary, the observed reduction in CRP levels suggests that dapagliflozin contributes to reversing processes related to inflammation which impact insulin sensitivity and cardiovascular disease risk.

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تقييم تأثير داباغليفلوزين على مستويات CRP لدى مرضى الداء السكري من النمط الثاني

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ملخص

الداء السكري من النمط 2 (T2DM) هو مرض مزمن ينتشر بشكل متزايد ويترافق مع زيادة خطر حدوث مضاعفات على مستوى الأوعية الدموية الصغيرة والكبيرة. هناك أدلة واضحة تثبت أن الداباغليفلوزين قد يخفض الالتهاب المزمن بالإضافة إلى تأثيره الخافض للغلوكوز، مما يمنع تطور المرض ومضاعفاته. تهدف هذه الدراسة إلى تقييم آثار الداباغليفلوزين على مستويات المشعر الالتهابي بروتين سي التفاعلي (CRP) لدى مرضى T2DM. تم تقسيم مرضى T2DM بشكل عشوائي إلى مجموعتين، تألفت المجموعة الأولى من 52 مريضاً الذين تلقوا جرعة يومية من الداباغليفلوزين كعلاج إضافي لخافضات سكر الدم الفموية الأخرى، في حين أن المجموعة الثانية (مجموعة الشاهد) تكونت من 60 مريضاً الذين تلقوا خافضات سكر الدم الفموية (ميتفورمين، سلفونيل يوريا، ثيازوليدين ديون، والغلبتينات). بعد مرور ستة أشهر، أظهرت نتائجنا تغييراً كبيراً هاماً إحصائياً في مستويات CRP بعد المعالجة بالداباغليفلوزين مقارنة مع الشاهد. على الرغم من أن معدل انخفاض CRP كان له دلالة إحصائية هامة عند المرضى المعالجين بجرعة 5 مغ و10 مغ، إلا أنه كان أعلى مع الجرعة الأخيرة. بالإضافة إلى ذلك، كان الانخفاض في مستويات CRP ذو دلالة إحصائية هامة عند كل من المرضى المضبوطين وغير المضبوطين، ولكنه كان أكبر عند المرضى غير المضبوطين. تم ملاحظة وجود ارتباط إيجابي غير هام من الناحية الإحصائية بين HbA1c وCRP عند بداية الدراسة (r: 0.21, p: 0.1)، وأثناء فترة المتابعة عند 3 أشهر (r: 0.10, p: 0.4) وعند 6 أشهر .(r: 0.08, p: 0.5) أظهرت دراستنا أن داباغليفلوزين له تأثير مفيد على المشعر الالتهابيCRP لدى مرضى T2DM.

الكلمات الدالة: داء السكري من النمط الثاني، داباغليفلوزين، البروتين سي التفاعلي، الالتهاب.

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In vitro Assessment of Antibacterial Activity and Potential Genotoxic Effect of Fruit Extracts of *Capparis spinosa* L. Plant

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ABSTRACT

This research was carried out to assess the minimum inhibitory concentration (MIC) and the genotoxic potential of ethanolic and aqueous (cold and hot) fruit extracts of *Capparis spinosa* L. (*C. spinosa*) plant against different types of bacterial strains. The antimicrobial effect of these extracts against the tested bacteria was investigated using broth microdilution method. The potential genotoxic effect was evaluated by ERIC-PCR technique. Results of the current study revealed that the MIC values of ethanolic fruit extract against the tested bacterial had a range of 12.5 mg/ml to 25 mg/ml. However, aqueous fruit extracts had an MIC with a range of 50 mg/ml to 100 mg/mL. The potential genotoxic activity of cold aqueous extract was determined according to the changes in ERIC-PCR profile of *E. coli* strain treated with extract in comparison to that untreated (negative control). Results of this study suggest the genotoxic effect of aqueous fruit extract on *E. coli*. Further research is required to assess and identify the biological molecules and their mechanisms in the context of the genotoxicity. In vivo genotoxicity assessment or with the presence of liver extract is recommended to evaluate the safety of using fruits for therapeutic purposes and a valuable nutrient source.

Keywords: *Capparis spinose*, antimicrobial activity, potential genotoxic effect, ethanolic fruit extract, aqueous fruit extract.

1. INTRODUCTION

Capparis spinosa plant is commonly known as a caper, plant belonging to genus *Capparis* of the family Capparidaceae. It is a perennial spiny bush that has fleshy leaves and big-white to pinkish-white flowers. This plant has a deep tap roots, woody stems; evergreen leaves, orbicular to elliptic, base rounded and apex mucronate, alternate. It hasa complete flower, showy, with four sepals, and four white to pinkish-white-colored petals, several long violet-colored stamens, with a single stigma. The small bud(caper)that grows to become a flower, which results in a fruit caperberry development with a delicate fruity flavor[1].

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The bush of *C. spinosa* is native to the Mediterranean region, it is a drought tolerant plant, mainly distributed in arid and semi-arid regions of the tropical and subtropical world [1,2]. The fruit is considered as a rich source of high-value nutrients. The *C. spinosa* plant is used in indigenous medicine to prohibit and/or relieve several of health issues such as kidney problems, obesity, hepatitis and diabetes. A wide range of pharmacological activities of various *C. spinosa* plant's parts have been described. These activities including antibacterial, cytotoxic, antiviral, anthelminthic, antifungal, antioxidant, anti-inflammatory, antiarthritic, chondroprotective, cardiovascular, respiratory, antidiabetic, antiallergic and antihistaminic, hypolipidemic, antipyretic, anticarcinogenic, hepatoprotective, immunomodulatory, diuretic, hypoglycemic and antihepatotoxic[1,2].

Antibacterial effect of fruit extracts of *C. spinosa* plant on different bacterial species has been studied previously[3-6]. Genotoxicity of buds aqueous extract and leaf aqueous extract of *C. spinosa* plant has previously been studied[7,8]. However, the genotoxic potential of fruit extract has not been previously evaluated. The current study was carried out to determine the minimum inhibitory concentration (MIC) of ethanolic and aqueous fruit (cold and hot) extracts of *C. spinosa* growing wild in Palestine, against different types of bacteria. In addition, to assess the genotoxic potential of cold aqueous fruit extract on *Escherichia coli* (*E. coli*) ATCC 25922 strain using enterobacterial repetitive intergenic consensus (ERIC)-PCR technique

2. RESULTS

2.1. Antimicrobial activity of C. spinosa fruit extracts

Results of the present study revealed that the ethanolic, aqueous fruit (cold and hot) extracts of *C. spinosa* were active against the studied bacterial strains. Ethanolic fruit extract exhibited higher antibacterial activity against both Gram-positive and Gram-negative bacteria than aqueous fruit (cold and hot) extracts. The studied bacteria were sensitive to ethanolic fruit extract concentrations ranging from 12.5 to 25 mg/ml. However, these tested bacteria were susceptible to the aqueous fruit (cold and hot) extracts concentrations ranging from 50 mg/mL to 100 mg/mL. The MIC profile of ethanolic, aqueous fruit (cold and hot) extracts of *C. spinosa* plant against different tested bacterial strains is presented in Table 1.

Table 1: MIC profile of ethanolic and aqueous fruit (cold and hot) extracts of *C. spinosa* plant against different types of bacterial species.

Bacterial strain	Fruit Extracts MIC±SD[a] (mg/ml)		
	Ethanolic	Cold water	Hot water
***S. aureus*(ATCC 6538P)**	12.5±0.0	50±0.0	50±0.0
MRSA (clinical isolate)	25±0.0	100±0.0	100±0.0
***E. coli* (ATCC 25922)**	12.5±0.0	50±0.0	50±0.0
***K. pneumoniae* (ATCC 13883)**	25±0.0	100±0.0	100±0.0
***B.subtillus* (ATCC 6633)**	1.25±0.0	100±0.0	100±0.0
***S. epidermidis* (ATCC12228)**	12.5±0.0	50±0.0	50±0.0

SD[a]: Standard deviation.

2.2. Evaluation of the genotoxic potential of C. spinosa aqueous fruit extract

The alterations in the extracted genomic DNA from both treated and untreated *E. coli* strain with different concentrations of cold aqueous fruit extract of *C. spinosa* plant were assessed and compared at the same time intervals using ERIC-PCR technique. In the current study, the ERIC-PCR profile revealed that the bands with an amplicon fragment size of approximately 300-bp length and 450-bp length were less intense or invisible in *E. coli* strain treated with dose 32.25 µg/ml of cold aqueous fruit extract of *C. spinosa* plant for 2h (Figure 1, lane 3), when these bands compared with the same bands that was produced in the negative control (Figure 1, lane C1). However, the ERIC-PCR profile also showed that the bands with an amplicon fragment size of about 1000-bp length and 450-bp length were more intense in *E. coli* strain treated with doses 62.5 µg/ml and31.25 µg/ml for 5h of cold aqueous fruit extract of *C. spinosa* plant(Figure 1, lanes 5 and6), in comparison with the same bands emerged in the negative control (Figure 1, lane C2).In addition, the

results of ERIC-PCR exhibited that the band which had an amplicon fragment size of approximately 650-bp length was higher intense in *E. coli* strain treated with dose 32.25 µg/ml of cold aqueous fruit extract for 5h (Figure 1, lane 6), when that band compared with the same band appeared in the negative control (Figure 1, lane C2).In this study, the ERIC-PCR profile showed that bands with an amplicon fragment size of about 1000-bp length and 650-bp length were less intense in *E. coli* strain treated with a dose 125 µg/ml of aqueous fruit extract of *C. spinosa* for 24 h (Figure 2, lane 7), when these bands compared with the same bands that appeared in the untreated control (Figure 2, lane C3). In addition, the band with an amplicon fragment size of about 650-bp length had less intensity in *E. coli* strain treated with a dose31.25 µg/ml of aqueous fruit extract of *C. spinosa* for 24 h (Figure 1, lane 7),when that band compared with the same band revealed in the negative control (Figure 1, lane C3). However, the band with a fragment size 450-bp length had a higher intense in *E. coli* strain treated with a dose 32.25 µg/ml of aqueous fruit extract of *C. spinosa* for 24 h (Figure 1, lane 9), in comparison with the same band seen in the negative control (Figure 1, lane C3).

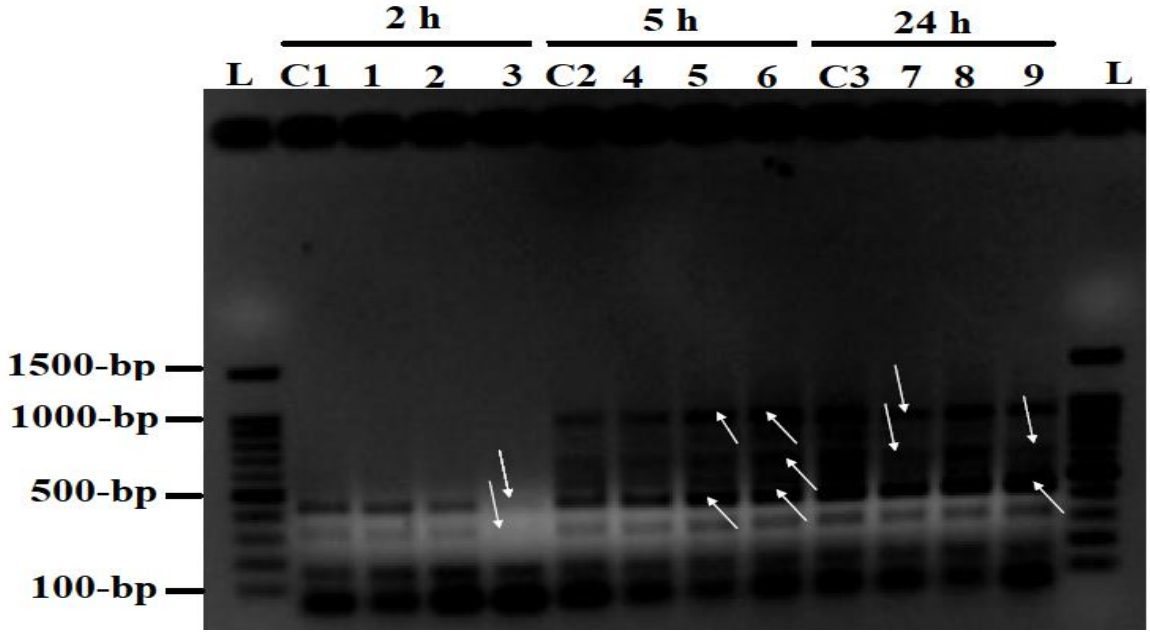

Figure 1: ERIC-PCR profile of *E. coli* strain treated with different concentrations of *C. spinosa* cold aqueous fruit extract at different time intervals and untreated (negative control). Lanes 1, 4 and 7 treated with 125 µg/ml; Lanes 2, 5 and 8 treated with 62.5 µg/ml; Lanes 3, 6 and 9 treated with 31.25 µg/ml of plant extract; Lanes C1, C2 and C3 are untreated (negative controls); Lanes L are 100-bp ladder).

3. DISCUSSION

Nowadays, researchers are seriously and continuously working on discovering and producing and synthesizing new drugs that act against bacterial infections. However, the bacterial strains are continuously opposing a challenge of this work by producing new strains that are resistant to the new produced drugs. In general, plants are considered a natural source that is rich with different by-products that harbor a potential antimicrobial activity against a broad range of pathogens[9,10]. In this study, ethanol and aqueous

(cold and hot) fruit extracts of *C. spinosa* plant were used to evaluate their antimicrobial properties against different bacterial pathogens by broth microdilution method. These pathogens included *S. aureus* (ATCC 6538P), MRSA (clinical isolate), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 13883) and *B. subtillus* (ATCC 6633).The results confirmed that ethanolic and aqueous (cold and hot) fruit extracts exhibited antibacterial activity against these studied microorganisms. Antimicrobial activity of fruit *C. spinosa* plant has been reported previously using different types of extracts[3-6].The antimicrobial activity is depending on the type of extracts. It has been reported previously that, flavonoid molecules are considered one of the major class of phenolic group, which have antimicrobial properties by inhibition of nucleic acid synthesis, cytoplasm membrane function and energy metabolism[11].

Nutraceutical molecules that are derived from different natural sources such as medicinal plants. Most of them have various medicinal properties and are declared to provide protection against many pathogens and various diseases if taken regularly. At the same time, studies which showed the safety evaluation and toxic activity of nutraceuticals have been very limited, so the safety of using of many of nutraceuticals cannot be assured[12]. In this study, the potential genotoxic effect of cold aqueous fruit extract of *C. spinosa* plant against *E. coli* strain was evaluated using ERIC-PCR technique. Reviewing the scientific literature showed that this is the first report studied the genetoxicity of aqueous fruit extract of *C. spinosa* plant on bacteria using PCR technique. Results of the current study showed that aqueous fruit extract of *C. spinosa*, altered ERIC-PCR profiles of *E. coli* strain treated with the aqueous fruit extract, in comparison with untreated *E. coli* strain (negative control). These results highly suggest the potential genotoxic effect of aqueous fruit extract from *C. spinosa* plant on *E. coli*. Results of this study are in agreement with results that have been published recently[8], which showed the potential genotoxic activity of the aqueous leaf extract of *C. spinosa* plant against *E. coli* using two molecular fingerprinting based techniques. However, our results are in contrast to that published previously[13,14], which exhibited that using *C. spinosa* is safe and there is insignificant scientific evidence regarding any adverse or toxic effects. On the other hand, other recent study showed that *C. spinosa* extracts had no potential toxicity effect at low doses but showed some toxicity at high doses. This is because that the crude extracts might have potential toxicity effect at higher concentrations[15]. Results of the this report are in contrast to findings of a study published previously[7], which revealed that aqueous extract of *C. spinosa* buds is non-genotoxic and the study showed the potential antimutagenic effect of the aqueous extract of *C. spinosa* buds against chromosomal aberrations in *A. cepa* root meristem cells induced by Ethyl Methane sulfonate. These variations in results of potential genotoxicity effect could be due to differences in plant part, test system and method used to evaluate potential genotoxicity effect. In a literature review, it was exhibited that plant extracts could be mutagenic and antimutagenic at the same time depending on the test system used to evaluate potential genotoxic effect. This demonstrates that it requires a category of tests or assays before any significant conclusion that can be given about the potential genotoxic effect[16].

4. MATERIALS AND METHODS

4.1. Plant collection

The caperberries were collected from a natural habitat in Tulkarm province, West Bank-Palestine, during July, 2020. The plant was identified by the plant taxonomist Dr. Ghadeer Omar, Department of Biology and Biotechnology, An-Najah National University, Palestine. The collected caperberries were washed thoroughly with water to get rid of soil and dust particles, and then were left in a shadow area away from light to minimize or reduce the possible loss of active ingredients. The air dried caperberries were finely powdered using an electric grinder to make them ready for extract preparation.

4.2. Plant extract preparation

4.2.1. Ethanolic fruit extract preparation

The ethanolic fruit extract was prepared according to method described previously with slight modifications[8]. Briefly, approximately 20 g of dried fruit powder was mixed in 200 mL of 70% ethanol; the mixture was incubated on orbital shaker at room temperature for 24h. After that, the mixture was filtered using three layers of medical gauze to get rid of large insoluble particles. Then, the obtained filtrate was centrifuged at 5,000 rpm for 10 min at 4°C to get rid of the small and fine particles. To possess a dried powder, the supernatant was left in incubator at 40°C. Finally, the obtained ethanolic fruit extract powder was stored in a refrigerator at 4°C. Before starting the experiments, a final concentration of 200 mg/mL of the ethanolic fruit extract powder was prepared in 10% Dimethyl Sulfoxide (DMSO) to be ready for assays.

4.2.2. Cold aqueous fruit extract preparation

The cold aqueous fruit extract was prepared according to method described previously with some modifications[8]. Briefly, approximately 20 g of dried fruit powder was mixed in 200 mL of cold (room temperature) sterile distilled water. Then, other following steps were as well as described in ethanolic fruit extract preparation. Before starting the experiments, a final concentration of 200 mg/mL of the cold aqueous fruit extract powder was prepared in sterile distilled water to be ready for assays.

4.2.3. Hot aqueous fruit extract preparation

The hot aqueous fruit extract was prepared as well as the same of the cold aqueous fruit extract preparation, except that the powder of dried fruits was added to a boiling water for 2 min.

4.3. Determination of MIC

MIC of plant extracts was determined by the broth microdilution method in sterile 96- wells microtiter plates according to the CLSI instructions[17]. The extracts were two fold-serially diluted in Mueller Hinton broth in the wells of plates. After that, to each well a 10^5 CFU/mL of a bacterial inoculum size was added. In this study, each plate was included different control wells, such as wells with Mueller Hinton broth only, DMSO and Mueller Hinton broth with microorganism inoculum, and plant extracts and Mueller Hinton broth without microorganism inoculum. Each experiment for each plant extract was conducted in duplicate. The covered plates were then incubated at 37°C for 24 h. The lowest concentration of the plant extract that completely inhibited bacterial growth was identified as the MIC value for that extract**.** The MIC values for the extracts were determined by visual inspection. The bacterial species included in this study were **Staphylococcus** *aureus* (*S. aureus* ATCC 6538P), Methicillin-resistant **S. aureus** (MRSA, clinical isolate), **Escherichia coli** (*E. coli* ATCC 25922), **Klebsiella pneumoniae** (*K. pneumoniae* ATCC 13883) and **Bacillus subtilis** (*B. subtillus* ATCC 6633).

4.4. Evaluation of the genotoxic potential of C. spinosa cold aqueous fruit extract

Few colonies from a 24 h old *E. coli* ATCC 25922 strain growth culture plated on Nutrient agar medium were sub-cultured under sterile conditions into a bottle containing 10-mL of nutrient broth, then the bacterial growth culture incubated at 37°C for 45 min with continuous shaking. After that, aseptically, 2 mL *E. coli* culture was added to each of four sterile bottles, each of which containing 25 mL broth medium. These bottles were incubated at 37°C for 1 hour with continuous shaking. The final concentration of cold aqueous fruit extract was 125 µg/mL in first bottle, 62.5 µg/mL in the second bottle and 32.25 µg/mL in the third bottle. However, the final concentration of cold aqueous fruit extract was 0.0 µg/mL in the fourth bottle which was considered as a negative or untreated control.

Two ml of bacterial sample was obtained from the E. coli culture treated and untreated with cold aqueous fruit extract after 2 h, 5 h, and 24 h. The genomic DNA of E. coli for these samples was extracted according to method described previously[18]. The concentration of genomic DNA for each sample was measured using a nanodrop

spectrophotometer (GenovaNano, Jenway) and the DNA samples were stored at -20ºC for ERIC-PCR technique.

The ERIC-PCR was performed using Primer ERIC1: 5`-ATG TAA GCT CCT GGG GAT TCA C-3` and Primer ERIC2: 5-AAG TAA GTG ACT GGG GTG AGC G-3` (Ventura et al., 2003)[19]. The assay conditions for PCR master mix preparation, DNA amplification and electrophoresis conditions were conducted according to method *described **previously**[8]*, except that the amount of DNA template was 20 ng for the samples collected at time interval 2 h, and 40 ng for samples collected at interval times 5 h and 24 h. *Variations in banding pattern profile following the amplified DNA extracted from E. coli strain treated with plant extracts were taken in consideration. The changes used for genotoxicity assessment including band intensity as well as gain or loss of bands [8,20,21].*

5. CONCLUSION

Findings of the current study showed that that fruit extracts of *C. spinosa*, especially ethanolic extract has antimicrobial activity and can inhibit the growth of different types of bacteria species. In addition, results of this study highly suggest the potential genotoxic effect of cold aqueous fruit extract prepared from *C. spinosa* plant on *E. coli*. Further research is required to assess and identify the exact biological molecules and their mechanisms in the context of the genotoxicity of this plant. In vivo genotoxicity assessment or genotoxicity assessment with the presence of liver extract is recommended to evaluate the safety of using fruits for therapeutic purposes and a valuable nutrient source.

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التقييم المخبري للنشاط المضاد للبكتيريا واحتمالية السمية الجينية لمستخلصات ثمارنبات القبار *Capparis spinosa* L.

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ملخص

تم إجراء هذا البحث لتقييم الحد الأدنى من التركيز المثبط (MIC) واحتمالية السمية الجينية لمستخلصات الثمار الإيثانولية والمائية (الباردة والساخنة) من نبات القبار *Capparis spinosa* L. (C. spinosa) ضد أنواع مختلفة من السلالات البكتيرية. تم استخدام طريقة التخفيف الدقيق المتسلسل من اجل معرفة تأثير المستخلصات لهذه الثمار انواع مختلفة من البكتيريا. كما وتم تقييم احتمالية السمية الجينية باستخدام تقنية قائمة على تفاعل البوليميراز المتسلسل.(ERIC-PCR) أظهرت الدراسة أن قيم الحد الأدنى من التركيز المثبط لمستخلص الثمار الإيثانولي ضد البكتيرياالمستخدمة في الفحص كانت ما بين 12.5 ملغ/مل إلى 25 ملغ مل. بينما قيم الحد الأدنى من التركيز المثبط لمستخلصات الثمار المائية كانت ما بين 50 ملغ/مل إلى 100 ملغ/مل. لقد تم تحديد احتمالية السمية الجينية لمستخلص الثمار المائي البارد وفقا للتغيرات على ملف تعريف ERIC-PCR لسلالة البكتيريا الإشريكية القولونية المعالجة بالمستخلص النباتي مقارنة بغير المعالجة (الشاهد السلبي). تشير نتائج هذه الدراسة أن مستخلص الثمار المائي البارد قد يكون له تأثير سمية جينية على الإشريكية القولونية. هناك حاجة إلى مزيد من البحوث لتقييم وتحديد الجزيئات البيولوجية وآلياتها في سياق السمية الجينية لهذا النبات. يوصى بتقييم السمية الجينية في الجسم الحي أو تقييم السمية الجينية مع وجود مستخلص الكبد لتقييم سلامة استخدام الثمار لأغراض علاجية أو كمصدر غذائي.

الكلمات الدالة: نبات القبار، نشاط مضاد للميكروبات، السمية الجينية، مستخلص الثمار الإيثانولي، مستخلص الثمار المائي.

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A Cross-sectional Study of the Catalase Genetic Polymorphism (-262 cytosine/ thymine) and Blood Catalase Activity among Jordanian Vitiligo Patients

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ABSTRACT

Vitiligo is brought on by functional melanocyte loss and manifests as white maculae that may cover the whole body's skin. There is a genetic background in the pathogenesis of vitiligo. Polymorphisms in different parts of catalase gene may affect the disease activity and result in less functional catalase, thus, accumulation of hydrogen peroxide, one of the oxidative factors that damage melanocytes. We evaluated the CAT 262 genetic polymorphism of vitiligo patients using the polymerase chain reaction (PCR) technique with at least one C and at least one T model. The study included 48 vitiligo patient and 51 control individuals. Family history of vitiligo was present in 27.1% of patients and autoimmune disease were diagnosed in 16.7% of patients. Three quarters of vitiligo patients (75.0%) reported that emotional stress was the major triggering factor for their disease. The CC genotype was predominant (56.2% in vitiligo patients and 62.7% in control) with no significant difference between the study groups (p=0.7). Catalase activity in blood was comparable between the study arms (159.1±21.6 MU/L in vitiligo patients and 151.3±25.4 MU/L in controls (p=0.15). We conclude that neither genetic polymorphism in CAT 262 C/T nor blood catalase activity is associated with vitiligo.

Keywords: Genetic Polymorphism, Blood Catalase, Vitiligo, CAT Gene (-262 cytosine/thymine), Jordanians.

INTRODUCTION

The most prevalent pigmentary condition, vitiligo, is brought on by functional melanocyte loss and manifests as white maculae that may cover the whole body's skin[1].

The etiology of vitiligo appears to be multifactorial, including mainly environmental, genetic, and immunologic factors which may synergistically cause melanocyte destruction[2]. The two main types of vitiligo are non-segmental vitiligo, which is the most prevalent form and often manifests as bilateral white patches, and segmental vitiligo, which has a unilateral distribution[3] and contributes to 5–16% of cases[4]. In 87% of cases the disease started before the age of 30 years and in 41.3% it started before the age of 10[3].

In Jordan, the prevalence of vitiligo in children was found to rise with age (0.45% 1 years, 1% 1-5 years, 2.1% 5-12 years). The prevalence of vitiligo was found to range from 0.06% to 2.28% in the general population and from 0% to 2.16% in juvenile populations. In 92.9% of vitiligo patients, non-segmental form was present[5].

Studies using histology and immunohistochemistry demonstrated that the afflicted skin region is devoid of melanocytes, despite their occasional presence[6]. High level of both organ- and non-organ-specific autoantibodies have been reported in the serum of vitiligo patients [7]. The antimelanocyte and antinuclear antibodies were shown to be higher in vitiligo patients as opposed to controls, furthermore, these antibodies levels were shown to

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correspond with disease activity[8]. These autoantibodies, which belong to the class G immunoglobulins, were also discovered in the lesional vitiligo epidermis' basal layer, together with deposits of complement component 3 (C3)[9]. Tyrosinase, tyrosinase-related protein-1 (TRP-1), TRP-2, Pmel17 (also known as gp100), the transcriptional factors SOX 9 and SOX 10, and the type 1 membrane receptor for melanin-concentrating hormone are the major melanocytic antigens (MCH-R1)[10,53]. Additionally, people with vitiligo have high frequencies of cytotoxic T lymphocytes that are reactive to melanocytes in their peripheral blood[11], capable of releasing type B granzyme, perforin, and IFN, while vitiligo epidermis exhibits perilesional T-cell infiltration[12]. It was shown that CD8+ T lymphocytes that have the characteristics of skin-homing, are oriented toward type-1 effector function, and are significantly cytotoxic being clustered around disappearing melanocytes[13].

Recent studies of the pathophysiology of vitiligo highlighted the significance of reactive oxygen species (ROS) and their function in melanocyte-intrinsic abnormalities as potential major triggers of the whole inflammatory cascade[14].In oxidative stress, superoxide is a primary oxygen radical produced when an oxygen molecule receives one electron. Superoxide dismutase (SOD) converts the superoxide to hydrogen peroxide (H_2O_2) that, in the presence of free ferrous iron, may produce hydroxyl radicals and exacerbate diseases[15]. Normally, the antioxidant system defends the cell from ROS, however, during oxidative stress, this system becomes unbalanced[16,54], in addition to H_2O_2 buildup brought on by environmental trauma like UVB exposure (290-320 mm)[17]. Together, autoimmunity and oxidative stress have a synergistic impact on melanocytes, causing cell death and depigmentation[18]. The immune system creates a long-lasting inflammatory environment where ROS build and damage nearby cells[19].

One of the antioxidant enzymes is catalase which is responsible of the conversion of hydrogen peroxide, one of ROS, to water and oxygen. A number of studies investigated the catalase activity in both plasma and epidermis of vitiligo patients with contradictory results[7,17, 20].

It has been suggested that a relation exists between genetic factors and susceptibility of vitiligo.

Genetic polymorphisms in different regions in CAT gene (a gene that encodes for catalase) have been studied in relation to vitiligo[21]. The gene for human catalase has been mapped to chromosome 11, band p13, and is split into 13 exons by 12 introns and spans[22]. Numerous polymorphisms have been described in the promoter, 5´ and 3´- untranslated regions (UTRs), exons and introns[23,24].

This study focuses mainly on the most common CAT gene polymorphism -262 cytosine/thymine (-262 C/T) in the promoter region in which C to T substitution occur at position -262 (db SNP ID: rs1001179) and that has been found to be associated with alteration in the CAT activities. The 262 C/T polymorphism may affect CAT transcription by modulation of the transcriptional factor binding position and increased basal CAT expression in various cell including erythrocytes[24]. The latter study investigated the relation between 262 C/T genetic polymorphism and the level of catalase in general, not in relation to any specific disease, and found that individuals carrying T allele have significant higher level of catalase in comparison with individuals carrying homozygote C allele[24]. On the contrary, another group of investigators showed that CC homozygotes had higher activity of CAT compared to those with CT or TT genotypes[25]. A later study found no association between the CAT262C/T polymorphism and CAT activity[26].

With regards to the association of CAT262C/T polymorphism and vitiligo in particular we found two studies, and both demonstrated lack of such association[17,27].

The aim of the study was dual: to assess the relation between the CAT 262 C/T polymorphism of and the susceptibility to vitiligo in Jordanian patients; in addition to the study of blood catalase activity in Jordanian vitiligo patients.

METHODOLOGY

Study design

This is a cross-sectional study that involved patients diagnosed with vitiligo and apparently healthy individual matched by age and gender who served as control.

Sample size

a) Using the online software https://clincalc.com/stats/samplesize.aspx, we have calculated the required sample size for detecting differences in blood catalase activity between vitiligo patients and found the size to be 24 per group based on the data from the previous publication[28]. However, we included 48 patients and 51 controls.

Clinical Setting and Patients

The vitiligo patients (cases) were recruited from the Dermatology Clinics at Al-Basheer Hospital related to Jordanian Ministry of Health and Kind Hussein Medical City related to the Royal Medical Services, Amman, Jordan over the period of May 1, 2015 to June 17, 2015. Vitiligo diagnosis was established by dermatologists using standard diagnostic criteria[3]. The active vitiligo was defined as the progression or appearance of new lesions in the last 3 months and the stable vitiligo was defined as the absence of new lesions or progression in the last 6 months, respectively[29].Healthy volunteers (control) were recruited from the outpatient clinics.

Inclusion criteria for vitiligo cases

1. Jordanian nationality.
2. Age between 18 and 60 years.

Exclusion criteria for cases

1. Any acute or chronic disease
2. Patient with other skin diseases

Inclusion criteria for controls:

1. Jordanian nationality
2. Age-matched (by decades) and gender-matched to control apparently healthy individuals.

Exclusion criteria for control

1. Any acute or chronic disease
2. History, either personal, or family, of any autoimmune diseases
3. No personal or family history of vitiligo.

Ethical consideration

The privacy and rights of the human participants were upheld throughout the investigation. The Institutional Review Boards' research ethical permissions were obtained from Jordanian Ministry of Health (approval number 4822 on March 10, 2015) and Royal Medical Services (approval number 3542 on February 25, 2015). Written informed consent form was provided to each patient, who was then requested to read and sign the form. The use of a code number for each participant rather than his name or file number were used to protect the anonymity and confidentiality of the information, and the potential study recruits were notified that participation in the study is voluntary and that they have the choice to decline without consequence.

Assessment of the CAT 262 polymorphism

The CAT 262 genetic polymorphism was evaluated using the polymerase chain reaction (PCR) with at least one C and at least one T model.

DNA extraction

Venous blood samples were collected from the patients and healthy subjects in K3 EDTA-coated tubes. The tubes were kept in icebox and DNA extraction was performed in the same day. Using the "Promega Wizard genomic DNA purification kit, Promega Corporation, USA" in accordance with the manufacturer's instructions, genomic DNA was isolated from the whole blood.

Assessment of DNA yield and purity

DNA was kept at -20° C until further investigation and the quality of the DNA was assessed using 0.5% agarose gel electrophoresis.

Genomic amplification

Using the forward and reverse primers, a 320 bp amplicon containing the targeted site, CAT 262 (rs1001179), was sequenced using PCR.

Measurement of blood catalase activity

Blood samples were collected by using EDTA tubes;

all samples were prepared after 4 hours of being collected to have consistency in our results. Erythrocyte hemolysis was induced by using saponin due to the high concentration of catalase in erythrocytes. Spectrophotometric assay was used to measure catalase activity in the blood based on the formation of stable complex between hydrogen peroxide and ammonium molybdate based on the method described by Goth (1992)[30]. In more details, one hundred µL of blood was dissolved it in 2 ml of 10% saponin solution. After finishing sample preparation 180 µL of lysate was taken and incubated it at 37° C for 60 second with 1.5 ml of 65 µmol/ml hydrogen peroxide, then 1.5 ml of 32.4 mmol/l ammonium molybdate was added to stop the enzymatic reaction resulting in a yellow solution that was measured against blank 3 at 405 nm.

The following equation was used to measure catalase activity:

Blood catalase activity (A) (kunits/l) = A (sample) - A (blank 1)/ A (blank 2) - A (blank 3) x 4 26 x 105

In which:

Blank 1: 1.5ml hydrogen peroxide + 1.5 ml ammonium molybdate + 1.5 PBS + 180 µL sample

Blank 2: 1.5ml hydrogen peroxide + 1.5 ml ammonium molybdate + 1.5 PBS

Blank 3: 1.5 ml ammonium molybdate + 1.5 PBS

Data management and statistical analysis

Continuous variables were reported as mean ± standard deviation (SD) and the comparison between the groups was conducted using independent sample t-test or one-way ANOVA test, as appropriate. Levene's test was utilized to examine the homogeneity of variance, and Kolmogrov-Smirnov test was employed to evaluate the normality of distribution. Chi-square or Fisher exact test was used to compare frequency of alleles and genotypes between the vitiligo and the control groups, where allele frequency = number of copies of an allele in a population /total number of all alleles for that gene in a population, and genotype frequency = number of individuals with a particular genotype in a population/total number of all individuals in a population. Genotype and allele frequencies were matched to expectation by Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium was assessed utilizing the formula:

$(a+b)^2$ with degree of freedom of 1,

where a is the frequency of one allele, and b is the frequency of the other allele. As a measure of the relationship between genotypes, odds ratios (OR) and their 95% confidence intervals (CI) were determined. The Mantel-Haenszel statistics were used to estimate the common odds ratios, their CI, and their p-values. For all comparisons, a p-value of 0.05 or less was regarded as statistically significant.

RESULTS

Study subject's description:

The study flow chart is shown in Figure1. Among 90 vitiligo patients approached initially, 42 were excluded, while among 121 healthy individuals approached initially, 70 were excluded from the study. The ultimate number of study participants was 48 vitiligo patients 51 control subjects.

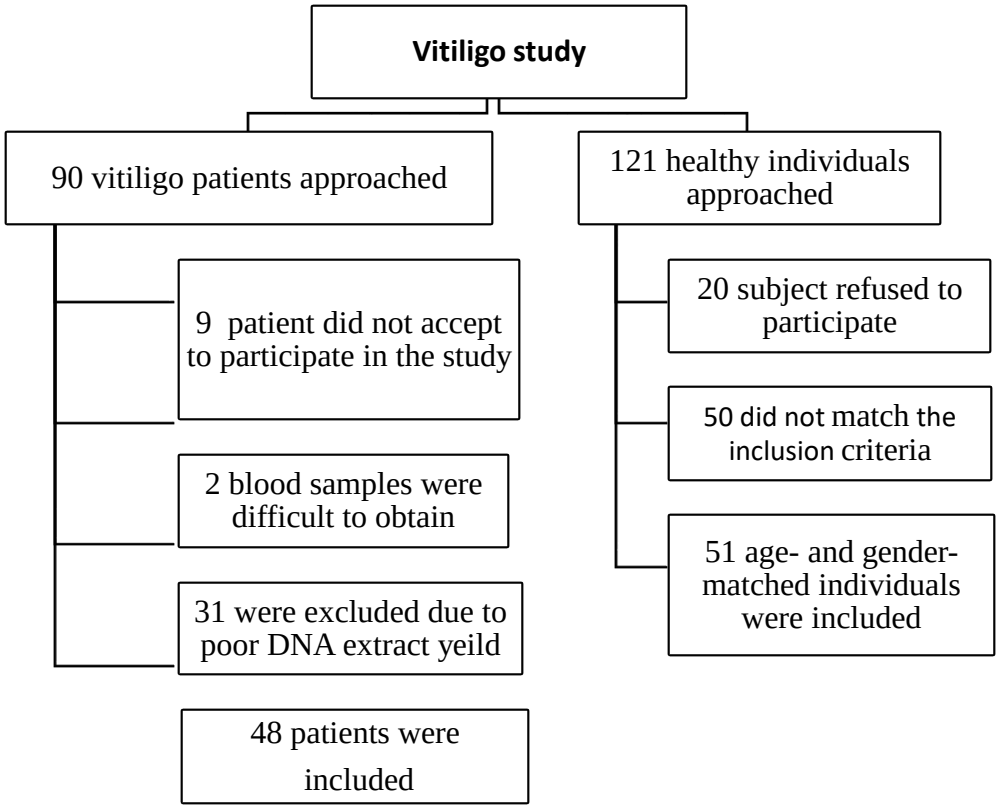

Figure 1. Study flow chart

Demographic and clinical characteristics of vitiligo patients:

Demographic and clinical characteristics of vitiligo patients are shown in Table 1. The mean age of vitiligo patients was 33.7 (±13.2) years.

The major proportion of vitiligo patients were females (72.9%)). Non-segmental vitiligo was diagnosed in majority of patients (93.7%), and the rest of patients had segmental vitiligo. Active disease was documented in more than half (53.9%) of cases. Three-quarters of patients (75.0%) reported that the emotional stress served as a triggering factor for their disease. Family history of vitiligo was present in 27.1% of vitiligo patients, while total of 16.7%of patients reported presence of autoimmune disease.

Blood catalase activity

The mean catalase activity in the blood (MU/L) did not differ significantly between the vitiligo patients (159.1±21.6) and the controls (151.3±25.4) (p=0.15). The catalase activity was also similar among the vitiligo patients with and without active disease (161.4±22.3 and 156.3±21.0, respectively, p=0.9). Furthermore, the catalase activity did not differ between vitiligo patients who received and who did not receive Psoralen plus ultraviolet A (PUVA) therapy in Table 2.

Table 1. Clinico-demographic characteristics of vitiligo patients (N=48)

Characteristic	
Age, mean (SD)	33.7 (13.2)
Gender, N (%)	
Males	13 (27.1)
Females	35(72.9)
Education level, N (%)	
Elementary	9 (18.87)
High school	27 (56.3)
Diploma	8 (16.7)
University	5 (10.4)
Localization of vitiligo, N (%)	
Segmental	3 (6.3)
Non-segmental	45 (93.7)
Disease activity, N (%) (assessed in 39 patients)	
Active	21 (53.9)
Stable	18 (46.1)
Presence of risk factors for vitiligo, N (%)	
Emotional stress	36 (75.0)
Chemicals exposure	3 (6.3)
Sun exposure	2 (4.2)
Others	7 (14.6)
Positive family history of vitiligo, N (%)	
Yes	13 (27.1)
No	35 (72.9)
Presence of autoimmune diseases, N (%)	
Thyroid disorder	7 (14.6)
Rheumatoid arthritis	1 (2.1)

Table 2. Comparison of blood catalase activity in vitiligo patients according to treatment with PUVA therapy

PUVA therapy	N*	Mean (±SD)	p**
Treatment-naive	12	156.2 (21.9)	0.9
<6 months treatment	13	157.9 (17.5)	
>6 months treatment	11	162.3 (27.4)	

*The total number of patients was less than 48 due to missing data

** by one-way ANOVA test

Genotype & Sequencing results

Figures 2-4 show sequencing results for the complementary strand of CAT gene. Data were analyzed by using Chromas program; one black band means CC genotype, one green band means TT genotype and 2 bands mean CT genotype.

As shown in Table 3, the most prevalent genotype in both vitiligo cases and controls was CC genotype (56.2% and 62.7%, respectively). There was no difference in all three genotypes distribution between the vitiligo patients and the controls (p=0.7). Similarly, the allele distribution did not differ between the vitiligo patients and the controls (p=0.6).

Table 3. Genotype and allele frequencies among vitiligo patients and controls

	Cases (N=48), N (%)	**Controls (N=51), N (%)**	**Total**	**p**
Genotype				
CC	27 (56.2)	32 (62.7)	59 (59.6)	
CT	18 (37.5)	15 (29.4)	33 (33.3)	
TT	3 (6.2)	4 (7.8)	7 (7.1)	0.7*
Allele				
C	72 (75.0)	79 (77.5)	151 (76.3)	
T	24 (25.0)	23 (24.0)	47 (23.7)	0.6**

* By Fisher exact test

** By independent-sample t-test

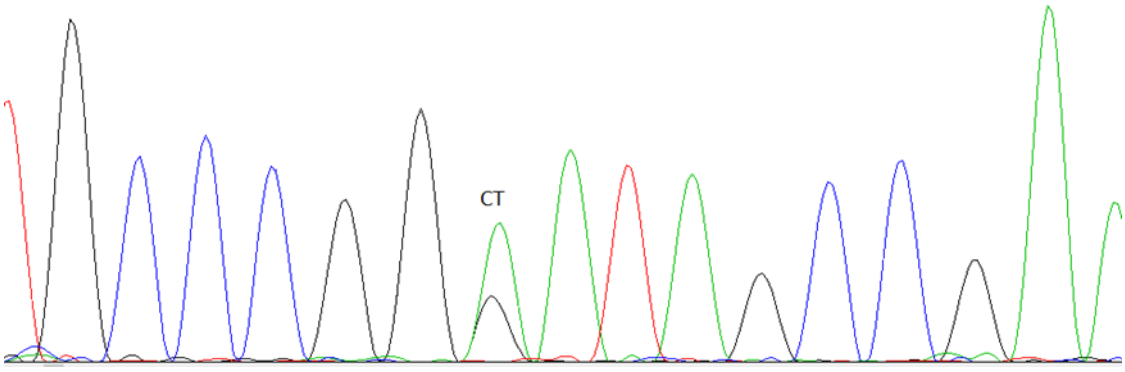

Figure 2. Genotype sequencing (CT)

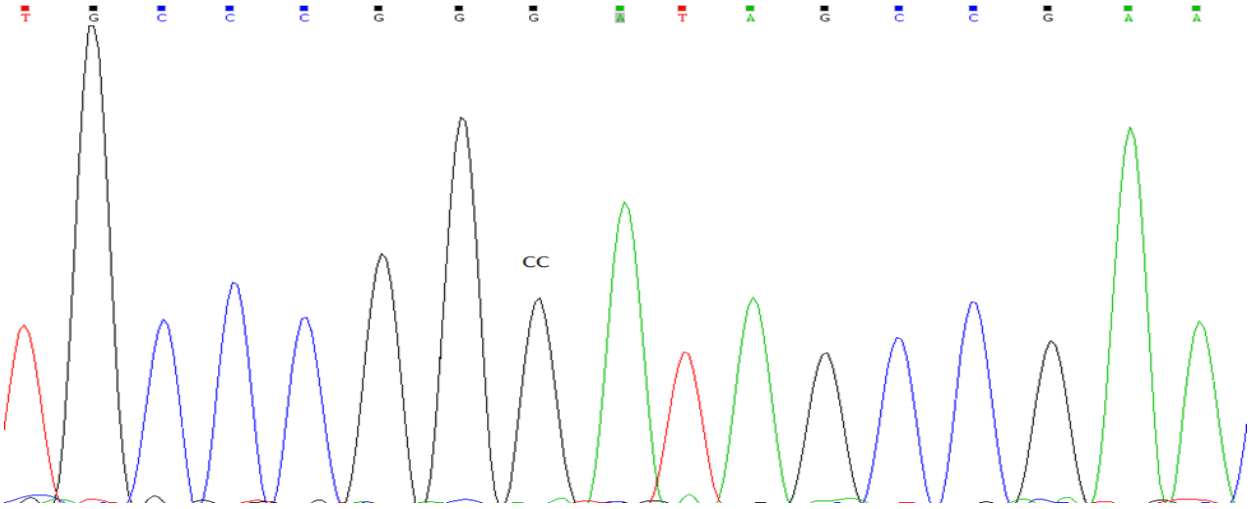

Figure 3. Genotype sequencing (CC)

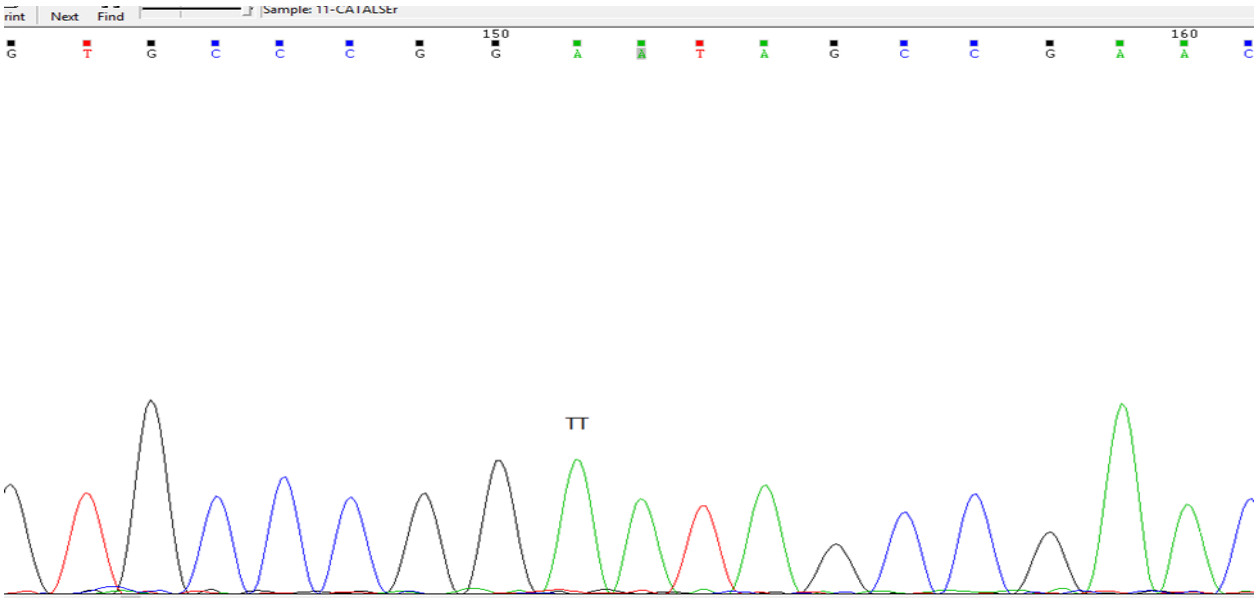

Figure 4. Genotype sequencing (TT)

As shown in Table 4, the occurrence of at least one T was not different between the vitiligo patients and controls (p=0.5), likewise, the occurrence of at least one C did not differ between the two study arms (p=0.8).

Table 4. Recessive and dominant genotype distribution among vitiligo patients and controls

		At least one T model		
Study group	**CC**	**CT+TT**	**Total**	**p**
Cases, N (%)	27 (56.3)	21 (43.7)	48 (100)	
Controls, N (%)	32 (62.7)	19 (37.3)	51 (100)	0.5
	At least one C model			
Study group	**CC+CT**	**TT**	**Total**	**p**
Cases, N (%)	45 (93.8)	3 (6.2)	48 (100)	
Controls, N (%)	47 (92.2)	4 (7.8)	51 (100)	0.8

DISCUSSION:

Importance of the study

Among the hypotheses for vitiligo, oxidative stress is considered as the initial pathogenic event in melanocyte destruction[7]. It was recently demonstrated that oxidative stress, in addition to producing multiple forms of melanocyte death, can induce the formation of the inflammatory cytokines, such as IL-6, IL-8, CXCL12, CCL5, CXCL10, and CXCL16 from stressed melanocytes or keratinocytes, resulting in initiation or promotion of the autoimmune reaction toward melanocytes[31].

It is widely accepted that there is a genetic background in the pathogenesis of vitiligo. A few studies were conducted in Jordan to investigate associations of SMOC2[32], NALP1[33], PTPN22 1858C/T[34], PTPN22 and SMOC2[35] and NLRP1[36genes, the latter two in relation to autoimmune thyroid disease. However, the association of catalase gene with vitiligo has not been investigated in Jordanian population, therefore, we decided to focus on the CAT gene polymorphism 262 C/T as it is less frequently reported than other CAT gene polymorphisms.it was previously shown that polymorphisms in catalase gene may affect the enzyme activity and result in less functional catalase and accumulation of hydrogen peroxide and, on the other hand, these polymorphisms were associated with vitiligo in some populations. However, it was suggested that the controversy concerning the CAT activity in vitiligo patients may be at least partially related to the polymorphisms in the catalase gene[31].A meta-analysis demonstrated that the 389 C/T polymorphisms in CAT were not associated with the risk of vitiligo in Asians and Turks, on the contrary, the CT genotype was proposed to be a genetic risk factor for susceptibility to vitiligo, while in Western Europeans the CC genotype might decrease the risk of vitiligo[37]. A number of studies demonstrated that CAT -89A/T, -262G/A, and -262T/C polymorphisms in the promoter region and -20T/C in 5′-untranslated region have detrimental effects on the catalase expression or function[21,24,38,39]. In both Chinese and Indian populations, CAT -89A/T variants were associated with a significant decrease in CAT activity and a genetic predisposition for vitiligo, especially in active and generalized vitiligo patients[40,41]. On the other hand, the CAT –262G/A variant showed no change in CAT activity or risk of vitiligo in Indian population[41]. Furthermore, in Northwestern Mexicans, CAT 419 C/T gene polymorphism was not informative, -89 A/T was associated with risk, while 389 C/T conferred protection against vitiligo along with AT haplotype. Additionally, although the serum CAT activity was lower in vitiligo patients, there was no association with any of the polymorphisms[42].

Previous studies demonstrated that the CAT 262 C>T polymorphism may affect the transcription of reporter genes and the binding of transcription factors [4,40,43]. Thus, the purpose of this study was to determine whether there is association between the CAT 262 polymorphism, as well as the blood catalase activity, with vitiligo in Jordanians.

Study findings

Our study showed that 27.1% of patient had family history of vitiligo, lower than reported in a retrospective analysis conducted in Saudi Arabia (42.8%)[43] but higher than in the previous study from Jordan (19.2%)[32] and the US and UK populations (6.1%)[44].

In our study, 16.7% of patients had autoimmune disease in line with the Saudi study where 90.5 % of patient had no history of autoimmune disease[43]. Our data regarding prevalence of thyroid autoimmune disease (14.6%) are comparable with another report where thyroid issues were present in 21.1% of vitiligo patients[45].

Genotype

Our results reveal that CC genotype was predominant in both study groups (58.3% in vitiligo patients vs. 62.7% in control). This frequency was somewhat in between the Hungarian and the English populations (Hungarian 43% in vitiligo patients and 36% in controls[17], English 72.3% in vitiligo patients and 75.1% in controls[27]), but the lack of difference in genotype frequency between the vitiligo patients and the controls was consistent among the three studies. Notably, a recent comprehensive meta-analysis and prioritization study to identify vitiligo associated coding and non-coding single-nucleotide variants (SNV) using web-based bioinformatics tools prioritized CAT gene, among 13 SNVs, from a set of 291 SNVs, as a candidate contributing to vitiligo pathogenesis[46].

Catalase activity

There is controversy regarding changes in serum catalase activity in vitiligo patients. Patients with active localized vitiligo showed significant decrease in the catalase levels when compared with healthy controls in studies conducted in Turkey [47,48], Nepal[20] and India[7] Notably, lower catalase activity was found in segmental vitiligo patients, whereas in non-segmental vitiligo patients the enzyme activity was normal[49]. More recently, significantly reduced catalase activity, along with other oxidant-antioxidant systems changes were found in serum of Chinese patients with nonsegmental vitiligo compared with healthy controls (HC)[31]. The current study found no difference in blood catalase activity between the vitiligo patients and the control individuals, in line with the studies from Hungary[17] Tunisia[50] and Turkey[51]. Furthermore, in a study from India, there was a significant decrease in serum catalase activity in the active phase group (p value=0.044) but not in the static group (p=0.095) in comparison with healthy group[7], however, our data did not support this observation. Notably, blood catalase activity in our patients was not affected by PUVA therapy.

Study limitations

Selection bias might occur as this research was a hospital-based cross-sectional study. Female participants were represented more than males and the gender interference with genotype prevalence cannot be excluded. Furthermore, since the data on triggering factors for vitiligo were retrospectively collected, this may increase the risk of recall bias, especially for events that date back to years ago like excessive sun exposure or emotional stress. Additionally, catalase activity was detected only in the blood, and not in the epidermis, which is more invasive but could be more accurately detect the oxidative processes at the local disease site. Genomewide association study, the best type of investigation to detect genetic loci involved in vitiligo, was not conducted.

CONCLUSION

Genetic polymorphism CAT 262C>T is not related to the incidence of vitiligo. There is no difference in blood catalase activity between vitiligo patients and healthy individuals, between patients with active and stable disease, or between patients receiving PUVA therapy and those without such treatment.

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عدم وجود ارتباط بين تعدد الأشكال الجيني الكاتليز (262السيتوزين>الثايمين) مع التعرض للبهاق بين الأردنيين: دراسة حالة مراقبة

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ملخص

يتم جلب البهاق عن طريق فقدان الخلايا الصباغية الوظيفية ويظهر على شكل بقع بيضاء قد تغطي جلد الجسم كله. هناك خلفية وراثية في التسبب في البهاق. تعدد الأشكال في أجزاء مختلفة من الجينات الكاتلاز قد تؤثر على نشاط المرض وتؤدي إلى تقليل وظيفة الكاتليز ، وبالتالي، تراكم بيروكسيد الهيدروجين، واحدة من العوامل المؤكسدة التي تضر الخلايا الصباغية، واحدة من العوامل المؤكسدة التي تضر الخلايا الصباغية. قمنا بتقييم تعدد الأشكال الجيني CAT 262 من مرضى تعدد الاشكال الوراثي البهاق باستخدام تقنية تفاعل البلمرة المتسلسل (PCR) مع واحد على الأقل سي ونموذج تي واحد على الأقل. وشملت الدراسة 48 مريض البهاق و51 عينة ضابطه. كان التاريخ العائلي للبهاق موجودا في 27.1 ٪ من المرضى وتم تشخيص أمراض المناعة الذاتية في 16.0 ٪ من المرضى. أفاد حوالي ثلاثة أرباع مرضى البهاق (75.0٪) أن التوتر النفسي كان العامل الرئيسي المسبب لمرضهم. كان النمط الجيني CC السائد (56.2% لمرضى البهاق و 62.7% للظابطه) مع عدم وجود فرق كبير بين مجموعة الدراسة .(P=0.7) كان نشاط الكاتليز في الدم متقارب بين أطراف الدراسة (159.1± 21.6 MU وحدة/لتر في مرضى البهاق و151.3 ± 25.4 وحدة/لتر في العينه الضابطه .(P=0.15) نستنتج أنه لا يرتبط تعدد الأشكال الجيني في CAT262 ولا نشاط الكاتليز في الدم بالبهاق.

الكلمات الدالة: تعدد الأشكال الجيني، الكاتليز في الدم، البهاق، الجين -Cat 262 سيتوسين، ثيامين، الاردنيون.

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Antibacterial Activity of Phytochemicals in *Ficus thonningii* Leaves Extracts Against Some Selected Pathogenic Bacterial Prevalent in Sickle Cell Anemia

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ABSTRACT

Sickle cell anemia (SCA) is caused by point mutation involving substitution of valine by glutamic acid, clinical cause of this is that SCA patients are immunocompromised hence, prone to bacterial infections. *Escherichia coli, Klebsiella. pneumoniae, Salmonella typhi, Streptococcus pneumoniae and Staphylococcus aureus* are some of the bacterial associated with SCA. Here we investigated the antibacterial activity of extracts of *Ficus thonningii* leaves used by ethnomedicinal practitioners in the management of infections in SCA patients. The antimicrobial activity was determined based on average diameter of zone of inhibition (AVDZI), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) while, structure of compounds FTH1 (Bergapten), FTH2 (Protocatechuic acid) and FTH3 (Methyl ferulate) were identified based on Nuclear Magnetic Resonance (NMR) analysis. The results indicated that the crude extracts of methanol (MCE), hexane (HCE), chloroform (CCE) and methanol-water (MWCE) had AVDZI of ≥ 12.1 ± 0.12 mm, ≥ 11.0 ± 0.00 mm, ≥ 10.2 ± 0.25 mm and ≥ 12.1 ± 0.21 mm respectively while the isolated compounds FTH1, FTH2 and FTH3 had AVDZI of ≥ 10.1 ± 0.10 mm, ≥ 9.1 ± 0.12 mm, ≥ 7.1 ± 0.24 mm respectively. The AVDZI results was tested for statistical significance using one way ANOVA and Tukey Posthoc test, and was considered significant at $p < 0.05$. Our findings suggested that *F. thonningii* leaves extracts including compounds isolated from them are potential antibacterial agent and justify their use as antibacterial prophylactics in the management of infections in SCA patients by ethnic people in Nigeria

Keywords: Antibacterial, *Ficus thonningii*, infections, sickle cell anemia, ethnomedicine.

1. INTRODUCTION

Infections in sickle cell anemia are caused by several factors and pathological effects of sickle cell anemia (SCA) most times create an environment that support infections [1]. Area of necrotic bones act as foci for infections in SCA patients, which becomes established through hematogenous spread [2]. Generally, people living with SCA are immunocompromised however, the disease shows different level of severity and manifestations in different patients at different stages owing to patient phenotype [1]. Also, SCA patients are predisposed to certain iatrogenic infections due to therapeutic interventions [3]. Infections has long been known as one of the causes of vaso-occlusive crisis because they promote leukocyte and Red Blood Cell (RBC) adhesion [1, 4]. Infections can possess more non-specific effects that increases the risk and likelihood of sickling, this includes fever with water loss due to sweating, anorexia, and nausea with reduced oral fluid intake, diarrhea and vomiting contributes to dehydration [5].

They exist a correlation between respiratory infection and acute chest syndrome [4, 6]. Research indicates that during infection changes occurs at cellular level that predisposes SCA patients to crisis, this change occurs locally and systematically in infected tissues, such that

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neutrophils, basophils and monocytes attracted to sites of inflammations produces cytotoxic proteins which generates reactive oxygen species that causes oxidative damage, which then promotes endothelial activation and cell adhesion [7]. Sickle cell anemia is prevalent among Black Africans, Afro-Caribbean, Mediterranean, middle east and some parts of India [1, 8] and infections contributes significantly to mortality and morbidity in SCA. *Salmonella spp., Staphylococcus spp., K. pneumoniae, E. coli, Enterobacter spp., Acinetobacter spp., Streptococcus spp., Serratia spp.,* are among prevalent bacteria implicated in SCA responsible for arrays of infections among which includes but not limited to septicemias, urinary tract infections, myositis, meningitis etc. [11].

The use of synthetic antibiotic prophylaxis though beneficial; poses risk on SCA patient thus, the use of antibiotic prophylaxis attempt to strike a balance between risk to the individual and the danger resistant organisms pose to the whole population [9]. Research indicates that 9% of SCA patients with *S. pneumoniae* infection had reduced susceptibility to penicillin [10] however, in those on prophylaxis, research suggest the rates may be much higher [1]. Therefore, it is necessary to evaluate natural products as possible alternative in prophylactic management of SCA patients since mostly third world countries bears the burden of SCA

Although, the antibacterial activity of *Ficus thonningii* leaves as well as some of the compounds isolated from them have been reported. However, in this study we profiled methanol (MCE), chloroform (CCE), methanol-water (MWCE) and n-hexane (HCE) crude extracts as well as compounds isolated from CCE and MWCE on *E. coli, K. pneumoniae, S. typhi, S. pneumoniae and S. aureus* isolated from SCA patients in vitro to understand the medicinal bases for its use as antibacterial prophylaxis by ethnomedicine practitioners in the management and treatment of infections in SCA patients in Ebonyi State, Nigeria.

2. MATERIALS AND METHODS

2.1 Sample collection, identification and preparation

The leaves of *Ficus thonningii* used in this study were collected from Echi-Aba in Echi-Aba Development Center, Ebonyi Local Government Area Council, Nigeria (latitude: 6º24'51.9"N, longitude: 8º07'34.1"E). The plant locally known by the ethnic people as Orgbu was identified and authenticated by a taxonomist at the Department of Applied Biology, Ebonyi State University as *Ficus thonningii* [12, 13]. The leaves were air dried and pulverized with the aid of both mechanical grinder and mortar then kept for further use in an air tight container. All chemicals used were Sigma Aldrich quality grade, melting point was determined using a Duran Thiele apparatus while the structure of isolated compound was determined using Bruker 500MHz spectroscopy. All bacteria used were local clinical isolates while the ampicillin used was Cikacillin® (Ampicillin Trihydrate BP 250 mg). Statistical (Tukey posthoc) analysis was done in triplicate using SPSS software version 20 and regarded as significant at $P < 0.05$. The results of statistical analysis were presented as mean ± standard error of the mean (SEM)

2.2 Extraction and purification

Sequential extraction of the plant part was successively carried out separately with solvents of increasing polarity: n-hexane, chloroform, methanol and methanol-water mixture (4:1). Pulverized leaves (10 kg) was weighed out and soaked in the appropriate solvents in order of increasing polarity for 72 hours. The mixture was filtered, the filtrate was concentrated using a rotary evaporator (Stuart RE 300/MS, UK) to one-tenth of its volume at ≤ 40°C [14]. Each dried extract was weighed in an analytical balance (OHAUS PX225D, USA) and stored at 4ºC.

2.3 Column and flash chromatographic separation

In each, 15 g of the crude extract was subjected to column chromatography and eluted with Hex- EtOAc (80:20, 70:30, 60:40, 50:50.), EtOAc (100%) and MeOH (100%) gradients. Slurry of silica gel 70-230 mesh (600 g)

was made with the eluting solvent and packed into the glass column. The tap was opened to allow excess solvent to drain off. The leaves extracts (15 g) was dissolved in the eluting solvent and packed on top of the silica gel slurry. As soon as the column began to condition, glass wool fiber was placed on top of the extract and the eluting solvent was added. Collection of the eluent was done with 50 mL and 100 mL conical flasks. Further elution was done with increasing concentration gradients. For the MeOH leaves crude extract, elution was carried out using DCM-EtOAc (80:20, 70:30), EtOAc (100%), EtOAc-MeOH (50:50) and MeOH (100%) gradients. For n-Hex leaves crude extract, elution was done with Hex-DCM gradients (60:40, 50:50), EtOAc (100%), EtOAc-MeOH (50:50), and MeOH-H_2O (80:20). For $CHCl_3$ leaves crude extract, elution was done with Hex-DCM gradients (60:40, 50:50), EtOAc (100%), EtOAc-MeOH (50:50), and finally with 100% MeOH. Elution of MeOH-H_2O leaves extract was carried out with DCM-EtOAc (80:20), EtOAc (100%), MeOH (100%). Fractions collected were monitored for purity by spotting on thin layer chromatographic (TLC) plates. The fractions were subjected to further separation and purification using column and flash chromatographic technique. The fractions in $CHCl_3$ leaves fraction were FTH1, and FTH2 whereas, FTH3 was extracted from the MeOH-H_2O. Collected fractions from column chromatography were further purified using a solvent system of PET-$CHCl_3$ (4:1) as the mobile phase and this revealed one major spot with minor spots, these fractions were further purified using flash chromatographic technique (silica gel, mesh 230-400, 30 g) prior to each NMR analysis. Elution was initially carried out with varying solvent mixture of PET-$CHCl_3$. Elution with solvent mixture of PET-$CHCl_3$ (5:1) yielded a major single spot on TLC with some minor impurities at the origin. The process was repeated and similar for all fractions. Concentration, drying and washing of the fractions severally with methanol afforded extracts labelled FTH1, FTH2 and FTH3. The pure fractions were further recrystallized three times, weighed and stored for use at 4^{o}C

2.4 Nuclear Magnetic Resonance

Both the 1D and 2D NMR spectral analyses were carried out using Bruker 500MHz NMR spectrometer. The samples were dissolved in the appropriate solvent prior to analysis. The structures were elucidated using a combined NMR technique of ^1H, ^{13}C, DEPT-135, TOCSY, COSY, HSQC and HMBC NMR

2.5 Evaluation of antimicrobial activity

2.5.1 Determination of diameter of zone of inhibition

Bacteria organisms were obtained from Alex Ekwueme Federal University Teaching Hospital Abakaliki. The bacteria isolates were tested for viability by resuscitating them in buffered peptone water afterward they were subcultured into nutrient agar medium and incubated at 37^{o}C for 24 hours. The organisms were then stored at 4^{o}C until when needed. Agar well diffusion techniques as described by Adeniyi *et al.* [15] was adopted for the study. 18 mL of Mueller Hinton agar (MHA) plates (Oxoid, England) were inoculated with 0.1 mL of an overnight broth culture of each clinical bacteria isolate (Equivalent to 3 x 10^7cfu/mL) McFarland (MF) standard [16] in sterile petri-dish. The seeded plates were rocked for uniform distribution of the bacteria isolates and allowed to set. Holes were bored on the plates using standard sterile cork borer of 6 mm diameters and equal volumes of the proposed antimicrobial agent (1000 µL) were transferred into the well with the aid of micropipette. The experiments were done in triplicate. The control experiments were setup with 1000 µL of 70% methanol in separate well. The plates were allowed to stand for one hour at room temperature to allow proper diffusion of the extract [17]. The plates were incubated at 37°C for 24 hours until marked decline in potency of antibacterial agent to inhibit the growth of the test bacteria was observed. Zone of inhibitions were measured in millimeter (mm) and the average values were calculated and recorded.

2.5.2 Determination of the minimum inhibitory concentration

The method described by Adebayo *et al.* [18] was used. The determination of the minimum inhibitory concentration (MIC) was carried out on extracts and bacteria isolates that showed sensitivity against the growth of the test organisms. The medium used was MHA solution which was prepared according to the manufacturer's standard of 38 g/1000 mL. In this study double strength was prepared by dissolving 38 g in 500 mL of distilled water, homogenized and 5 mL was dispensed into 40 sets of McCartney bottles and sterilized in an autoclave at 121°C for 15 min. The agar was allowed to cool to approximately 45°C and each graded solution was then poured into Petri-dishes and allow to solidity for one hour. Extracts concentration of 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 mg/mL were prepared by serial dilution. Each plate was divided into 4 (four) equal section and labeled accordingly. The 5 mm diameter paper discs were placed aseptically into each labeled section of the plate using sterilized forceps. With an automatic micropipette, 0.1 mL of each bacterial suspension was taken and transferred aseptically into each appropriate pre-labeled paper disc on the agar plates. The plates were incubated for 24 hours at 37°C after which they were observed for growths or death of the test organisms. The lowest concentration inhibiting growth was taken as the minimum inhibitory concentration (MIC).

2.5.3 Determination of the minimum bactericidal concentration (MBC)

The determination of MBC was carried out by preparing 40 sets of plates of Mueller Hinton agar and sterilized. The paper discs in all the plates from MIC tests were reactivated, using a mixture of 0.5% egg lecithin and 3% Tween 80 solution. The reactivated organisms were subcultured into appropriately labeled quadrants of the sterilized Mueller Hinton agar plates. The organisms were uniformly streaked on labeled quadrants using wire loop. The organisms were incubated at 37°C for 24 hours, after which growth were observed and recorded. The MBC was the quadrant with lowest concentration of the extract without growth.

3. RESULTS AND DISCUSSIONS

3.1 Results of structural elucidations

FTH1: - Bergapten

White crystalline solid; R_f 0.68; melting point 188-190°C [19]; yield 0.13 %. ^{13}C (500MHZ, $CDCl_3$): 161.30 ppm (C-8), 158.36 ppm (C-11), 152.67 ppm (C-9), 105.09 ppm (C-1), 144.79 ppm (C-2), 149.55 ppm (C-4), 139.33 ppm (C-6), 112.58 ppm (C-3), 112.48 ppm (C-7), 106.32 ppm (C-5), 93.78 ppm(C-10), 60.06 ppm (C-12).^1H (500MHZ, $CDCl_3$): 8.16 ppm (H-6), 7.59 ppm (H-2), 7.13 ppm (H-10), 7.02 ppm (H-1), 6.28 ppm (H-7), 4.27 ppm (H-12). The ^{13}C, ^1H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for FTH1 including its physical characterizations matched those of Bergapten and was thus assigned [20, 21].

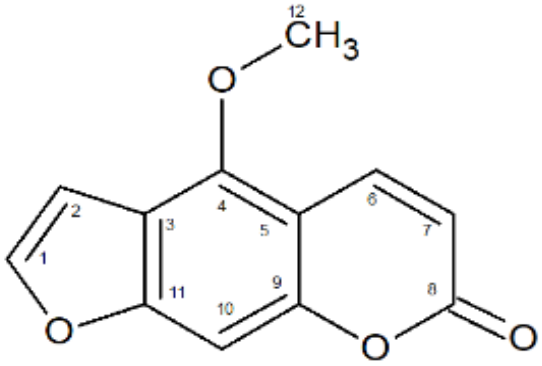

Figure 1. This figure depicts structure of Bergapten isolated from *F. thonningii* leaves

FTH2: - Protocatechuic acid

Gray crystalline solid; R_f 0.72; melting point 221-223°C [22]; yield 0.16 %. ^{13}C (500 MHZ, D_2O): 177.94 ppm (C-7), 150.04 ppm (C-3), 146.07 ppm (C-4), 131.39 ppm (C-5), 125.24 ppm (C-1), 119.63 ppm (C-2), 118.12 ppm (C-6). ^1H (500 MHZ, D_2O): 7.42 ppm (H-2), 7.39 ppm (C-6), 6.92 ppm (H-5). The 13C, 1H, DEPT-135, TOCSY,

COSY, HSQC and HMBC spectra including physical characterizations for FTH2 matched those of protocatechuic acid and was thus assigned [23]

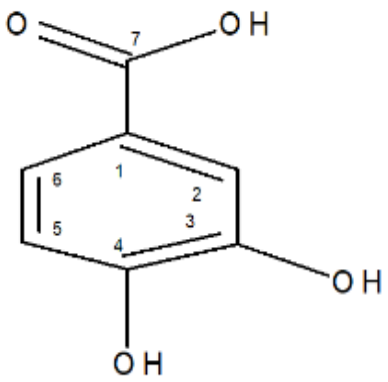

Figure 2. This figure depicts structure of protocatechuic acid isolated from *F. thonningii* leaves

FTH3: - Methyl ferulate

Yellowish brown crystal, Rf: 0.83, melting range: 63 - 65 °C [24], mass: 14.68 g yield 0.15%. ^{13}C (500MHZ, $DMSO_{-d6}$): 167.09 (C-9), 149.36 (C-4), 147.91 (C-3), 145.10 (C-7), 125.55 (C-1), 123.12 (C-6), 115.50 (C-8), 114.19 (C-5), 111.29 (C-2), 55.71 (3-OCH3), 51.22 (9-OCH3). ^{1}H (500MHZ, $DMSO_{-d6}$): 7.57 (H-7), 7.32 (H-6), 7.12 (H-2), 6.80 (H-5), 6.50 (H-8), 6.46 (4-OH), 3.81 (3-OCH_3), 3.70 (9-OCH_3). The ^{13}C, ^{1}H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for FTH3 including its physical characterizations matched those of methyl ferulate and was thus assigned [24].

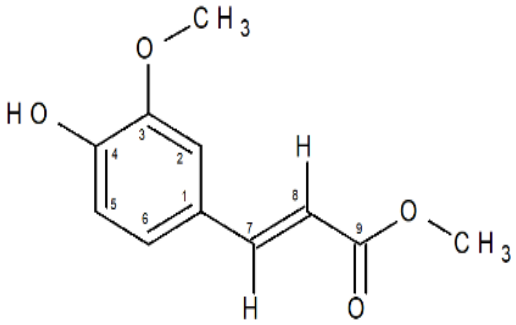

Figure 3. Methyl ferulate isolated from *F. thonningii* leaves

3.2 Results of Average diameter zone of inhibition

Table 1. Average diameter of zone of Inhibition for *F. thonningii* extracts (mm)

Extracts, and Drug	*Escherichia coli*	*Klebsiella pneumonia*	*Salmonella typhi*	*Streptococcus pneumoniae*	*Staphylococcus aureus*
MCE	16.3±0.30[a]	20.0±0.00[a]	12.1±0.12[a]	20.1±0.21[a]	18.0±0.15[a]
HCE	13.1±0.15[b]	19.2±0.20[b]	11.0±0.00[b]	20.3±0.23[a]	16.2±0.31[b]
CCE	12.3±0.15[c]	14.2±0.20[c]	10.2±0.25[c]	18.3±0.16[c]	15.1±0.13[c]
MWCE	14.1±0.1[b]	17.0±0.00[d]	12.1±0.21[a]	18.1±0.10[c]	18.2±0.23[a]
FTH1	10.1±0.10[e]	13.2±0.10[e]	11.0±0.00[b]	20.1±0.10[a]	13.0±0.00[e]
FTH2	9.1±0.12[f]	12.1±0.13[f]	10.2±0.09[c]	15.1±0.18[f]	12.1±0.21[e]
FTH3	9.0±0.00[f]	11.2±0.20[g]	10.0±0.00[c]	13.1±0.19[g]	7.1±0.24[g]
Ampicillin	19.1±0.12[h]	23.0±0.09[h]	17.2±0.17[h]	28.1±0.12[h]	25.2±0.17[h]

* a, b, c, d, e, f, g and h indicate the level of significance of the difference between different agents and Ampicillin as obtained from SPSS statistics Tukey Posthoc test. Distinct letters in the same column indicates significant difference ($P < 0.05$) while two letters of the same identity indicate non-significant difference ($P > 0.05$). **MCE** = methanol crude extract; **HCE** = hexane crude extract; **CCE** = chloroform crude extract; **MWCE** = methanol-water crude extract; **FTH1 =** bergapten; **FTH2** = protocatechuic acid; **FTH3** = methyl ferulate

Average diameter of zone of inhibition was used to evaluate the antibacterial activity of the antibacterial drug candidates and ampicillin. The results were presented as mean ± SEM. From Table 1, it was shown that the extracts and isolated compounds of *F. thonningii* showed viable activity against both gram positive (*S. aureus* and *S. pneumoniae*) and gram negative (*K. pneumonia, E. coli* and *S. typhi*) bacteria used in the study. The antibacterial activity of both the isolated compound and the crude extracts were significantly different from those of ampicillin at $P < 0.05$ i.e. the inhibitory activity of each antibacterial agent was significantly different from that of ampicillin. Consequently, bacteria used in the study were susceptible to both the antimicrobial drug candidate and ampicillin.

The antibacterial activity of *Ficus species* has been reported [18]. Similarly, the antibacterial activity of FTH1 [25, 26] has been validated while antibacterial and synergistic interaction of FTH2 with some antibiotics against resistant pathogens has been profiled [27]. Also, FTH3 is a novel natural antibacterial agent with strong activity and low toxicity [28, 29]. Generally, the antimicrobial properties of plant extracts are attributed to the presence of phytochemicals [30] this suggest that the observed presence of compounds FTH1, FTH2 and FTH3 are responsible for the antibacterial activity of *F. thononngii* leaves extracts. The observed antibacterial activity of *F. thonningii* [31], corroborate the listing of *Ficus species* as medicinal plants commonly used in ethnomedicine in Africa [32]

3.3 Results of Minimum inhibitory concentration

Table 2. Minimum inhibitory concentration of *F. thonningii* leaves extracts (mg/mL)

Extracts, and Drug	*Escherichia coli*	*Klebsiella pneumoniae*	*Salmonella typhi*	*Streptococcus pneumoniae*	*Staphylococcus aureus*
MCE	1.000	0.250	4.000	4.000	2.000
HCE	4.000	1.000	8.000	2.000	8.000
CCE	8.000	4.000	8.000	8.000	16.000
MWCE	2.000	2.000	4.000	8.000	4.000
FTH1	16.000	8.000	8.000	4.000	16.000
FTH2	32.000	16.000	8.000	32.000	16.000
FTH3	32.000	32.000	32.000	32.000	64.000
Ampicillin	0.250	0.500	1.000	0.250	0.125

MCE = methanol crude extract; **HCE** = hexane crude extract; **CCE** = chloroform crude extract; **MWCE** = methanol-water crude extract; **FTH1 =** bergapten; **FTH2** = protocatechuic acid; **FTH3** = methyl ferulate

From Table 2, with respect to *E. coli* the MIC of MCE (1.000 mg/mL), HCE (4.000 mg/mL), CCE (8.000 mg/mL) and MWCE (2.000 mg/mL) were slightly comparable to ampicillin (0.250 mg/mL). Similarly, it was observed that MCE inhibited the growth of *K. pneumoniae, S. typhi, S. pneumoniae, and S. aureus* at 0.250 mg/mL, 4.000 mg/mL, 4.000 mg/mL and 2.000 mg/mL concentrations respectively. Comparatively FTH3 had the highest minimum inhibitory concentration values for all analyzed bacteria indicating high dose requirements in vitro. MCE, HCE and MWCE inhibited the growth of *K. pneumoniae* at lower concentrations comparable to ampicillin.

Patients with homozygous sickle cell (SS) disease are at increased risk of infection with *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, *Salmonella* spp, *Escherichia*

coli and *Klebsiella* spp [33, 34, 35] therefore, the ability of the extracts of *F. thonningii* and its isolated compounds to inhibit the growth of *E. coli, K. Pneumoniae, S. typhi and S. pneumoniae* even at lower concentrations validated the use of *F. thonningii* in the management of infections in SCA patients in Southeast Nigeria.

3.4 Results of Minimum Bactericidal concentration

Table 3. Minimum Bactericidal concentration of *F. thonningii* leaves extracts (mg/mL)

Extracts, and Drug	*Escherichia coli*	*Klebsiella pneumonia*	*Salmonella typhi*	*Streptococcus pneumoniae*	*Staphylococcus aureus*
MCE	2.000	1.000	8.000	8.000	2.000
HCE	8.000	2.000	16.000	4.000	16.000
CCE	16.000	8.000	16.000	16.000	32.000
MWCE	4.000	4.000	8.000	16.000	4.000
FTH1	32.000	16.000	16.000	8.000	32.000
FTH2	64.000	32.000	16.000	64.000	32.000
FTH3	64.000	64.000	NA	64.000	NA
Ampicillin	0.500	1.000	2.000	1.000	0.250

NA = No activity at coverage concentration; **MCE** = methanol crude extract; **HCE** = hexane crude extract; **CCE** = chloroform crude extract; **MWCE** = methanol-water crude extract; **FTH1 =** bergapten; **FTH2** = protocatechuic acid; **FTH3** = methyl ferulate

The minimum bactericidal concentration is the lowest concentration of an antimicrobial agent required to kill a bacterium [36]. The MBC demonstrates the lowest level of antimicrobial agent that results in microbial death. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC [37]. From Table 3, it was shown that the MCE had the lowest MBC values when compared with other extracts of *F. thonningii* this was followed by MWCE. Methanol as an extracting solvent has high extractability and its polarity works on many phytochemicals including polar and nonpolar Phyto-constituents. FTH1 and FTH2 were compounds isolated from CCE of *F. thonningii* while FTH3 was identified from its methanol-water extract. Therefore, it is expected that the improved MBC (antibacterial activity) of MCE was probably due to compounds contained in its methanol and methanol-water extracts that function synergistically with other phytocompounds toward improved antibacterial activity.

The Burden and spectrum of bacterial infections among children living with SCA suggest that *Salmonella spp., Staphylococcus spp., K. pneumoniae, E. coli, Enterobacter spp., Acinetobacter spp., Streptococcus spp., Serratia spp.*, were responsible for 28.1%, 18.8%, 17.7%, 10.4%, 5.2%, 4.2%, 4.2%, and 4.2% of infections respectively and most cases of septicemias were reportedly caused by *Staphylococcus spp.* (24.6%), *Salmonella spp.* (24.6%) and *Klebsiella pneumoniae* (16.9%) also, *E. coli* led to majority of cases of urinary tract infections (53.8%) and *Salmonella spp.* were responsible for all cases of myositis while *S. pneumoniae* was responsible for two cases of meningitis [38]. Since previous report suggest that ethnomedicinal plant-based antibacterial agent are potent in the treatment of infections caused by *E. coli, S. aureus* and *S. pneumoniae* and several study support its broad-spectrum antibacterial activity [39, 40]hence, indicating the efficacy of ethnomedicine in the treatment of infections. Therefore, these bacteria even at very low extract

concentrations were susceptible to *F. thonningii* leaves hence, the use of *F. thonningii* leaves extract as a natural product prophylactic may possibly reduce the occurrence of these infections in SCA patients and thus possibly may also ameliorate the condition of sickle cell patients with respect to recurrence of painful episodes that occurs due to bacterial infections

CONCLUSION AND RECOMMENDATIONS

The antibacterial activity of the leaves extracts of *F. thonningii* was profiled against *E. coli, K. pneumoniae, S. typhi, S. pneumoniae and S. aureus* because of the prevalence of these bacterial and its infections among people living with SCA. The observed diameter of zone of inhibition showed that the crude extracts of the plant parts and compounds isolated from them showed average diameter of zone of inhibition comparable to those of ampicillin while the analyses of the MIC and MBC showed that the extracts inhibited the growth of these bacteria even at low concentrations thus, supporting the ethnomedicinal use of this plant in the treatment of infections in children living with SCA. Hence, *F. thonningii* should further be profiled in vivo for use in the management of infections in SCA patients. Clinical trials of compounds isolated from *F. thonningii* including those reported herein should be considered both as antibiotic-enhancer and prophylactics in the management of infections in SCA patients.

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Competing interest disclaimer

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النشاط المضاد للبكتيريا للمواد الكيميائية النباتية في أوراق *Ficus thonningii* المستخلصات ضد بعض البكتيريا المسببة للأمراض المنتشرة في فقر الدم المنجلي

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ملخص

تحدث فقر الدم المنجلي عن طفرة نقطية تتضمن استبدال حمض الغلوتاميك بالفالين، والسبب السريري لهذا هو أن مرضى SCA يعانون من نقص المناعة، وبالتالي يكونون عرضة للعدوى البكتيرية. الإشريكية القولونية، كليبسيلا. الرئوية، السالمونيلا التيفية، العقدية الرئوية والمكورات العنقودية الذهبية هي بعض البكتيريا المرتبطة بـ .SCA. هنا قمنا بالتحقيق في النشاط المضاد للبكتيريا لمستخلصات أوراق Ficus thonningii التي يستخدمها ممارسو الطب العرقي في إدارة العدوى في مرضى .SCA. تم تحديد النشاط المضاد للميكروبات بناءً على متوسط قطر منطقة التثبيط (AVDZI)، والتركيز المثبط الأدنى (MIC) والحد الأدنى لتركيز مبيد الجراثيم (MBC) بينما، بنية المركبات FTH1 (Bergapten)، FTH2 حمض (Protocatechuic و FTH3) ميثيل (ferulate) بناءً على تحليل الرنين المغناطيسي النووي .(NMR) أشارت النتائج إلى أن المستخلصات الخام للميثانول (MCE) والهكسان (HCE) والكلوروفورم (CCE) وميثانول الماء (MWCE) تحتوي على AVDZI بمقدار ≤ 12.1 ± 0.12 مم، ≤ 11.0 ± 0.00 مم، ≤ 10.2 ± 0.25 مم و ≤ 12.1 ± 0.21 ملم على التوالي بينما كان للمركبات المعزولة FTH1 و FTH2 و AVDZI FTH3 بمقدار ≤ 10.1 ± 0.10 ملم، ≤ 9.1 ± 0.12 ملم، 7.1 ± 0.24 ملم على التوالي. تم اختبار نتائج AVDZI من أجل الدلالة الإحصائية باستخدام اختبار ANOVA أحادي الاتجاه واختبار Tukey Posthoc، واعتبرت مهمة عند $p < 0.05$. تشير النتائج التي توصلنا إليها إلى أن مستخلصات أوراق F. thonningii بما في ذلك المركبات المعزولة منها هي عامل مضاد للجراثيم محتمل وتبرر استخدامها كعلاج وقائي مضاد للبكتيريا في إدارة العدوى في مرضى SCA من قبل الأشخاص العرقيين في نيجيريا.

الكلمات الدالة: مضاد للجراثيم، اللبخ thonningii، الالتهابات، فقر الدم المنجلي، الطب العرقي.

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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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