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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 (Pharmaceutical Technology, Pharmaceutics, Biopharmaceutics, Pharmacokinetics, Pharmaceutical/Medicinal Chemistry, Computational Chemistry and Molecular Drug Design, Natural Products Chemistry, Pharmacognosy, Phytochemistry, Pharmacology, Pharmaceutical Analysis, Pharmacy Practice, Clinical and Hospital Pharmacy, Pharmacogenomics, Bioinformatics and Biotechnology of Pharmaceutical Interest). The Journal publishes original research work either as a Full Research Paper or as a Short Communication. Review Articles on a current topic in Pharmaceutical Sciences are also considered for publication by the Journal. Now we are listed in C.A., Index Copernicus, Scopus…etc.

The Editorial Team wishes to thank their colleagues who have submitted the fruits of their labors to (*JJPS*). If you have any constructive criticism, please do not hesitate to contact us at **jjps@ju.edu.jo.** We hope that your comments will help us make the (*JJPS*) even better and appealing to all our readers.

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

Letter from the Editor-in-Chief

Two decades ago after the PhD , it was my great pleasure submitting one of my early articles to the Saudi Pharmaceutical Journal (ISI indexed) as the Editor-in-Chief –at that time- was one of my significant professors, being the top of my class, I had great expectations. Unfortunately, the paper was not accepted and so the first lesson learned: it is not only the quality of the research, but also the originality and of interest to the audience!

This is a call for all colleagues working in the pharmaceutical sciences fields to select Jordan Journal of pharmaceutical Sciences (JJPS) as a good choice for their publications. JJPS is a SCOPUS (Q3) indexed journal working hard forward being one of the Clarivate analytics (web of science) journals soon.

Going through the previous issues of the JJPS gives the reader a perception of purely chemical, technical, and pharmacological specialized submissions, in which the new editorial board encourages all researchers as well as post graduate students to submit their work in all pharmaceutical sciences' fields including pharmaceutical/medicinal chemistry and microbiology, biotechnology and industrial pharmacy, instrumental analysis, phytochemistry, clinical pharmacy and pharmaceutical care, 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.

JJPS will have a new start this Jan 2020 in which the new editorial board agreed to publish four issues per year with up to ten articles per issue. Hence, researchers will be able to publish their work as fast as possible. Furthermore, there will be special issues for some well recognized local conferences and scientific gatherings in the field of pharmacy in order to encourage local scientists and their students.

Finally, it really is a great honor to be the new Editor-in-Chief for JJPS. We are keen on continuity of the distinguished work of my previous colleagues since 2006, ensuring the same quality of work where each submitted article will be reviewed blindly by **at least** 2 reviewers in order to have an objective decision in this regard, concentrating more on scheduling time for each review with no delay. One last point worth mentioning that this issue is the first with our electronic ISSN number; a step forward for a complete electronic process in the future.

Prof Ibrahim Alabbadi
Editor-in-Chief

Editorial Commentary

Dear researchers,

Nowadays, the world is facing a real pandemic that affects people all over the world. With intense anticipation for a year, a break of good news from vaccine developers became a reality. People are now looking towards the pharmaceutical industry as their salvation. There is still too much to do; expecting from industry and regulatory bodies to produce effective and safe vaccines to combat the Covid-19 pandemic.

There has been immense development in the field of pharmaceutical industry starting from Pharma 4rth industrial revolution that created more advanced manufacturing practices. As well as, new technologies such as single use system for biotechnology manufacturing and continuous manufacturing lead to more innovative and consistent manufacturing of drugs and biological products. When implemented, these technologies reduce manufacturing failures that might cause supply disruptions and drug shortages. This would lead to more reliable supply of drug and biological products.

As researchers, this pandemic creates an opportunity for us to contribute to the world's efforts to face this challenge. As the spread increases, we recognized the gaps in the research field and development globally. This issue was evident during this difficult pandemic time. The development and approval of new technologies, biotechnology products, patent protected medicines should never been an excuse for shortages in medicines supply worldwide. Researchers should be engaged in more research towards the development of biosimilar products, generic medicines, and even conventional vaccines especially in under developed countries. Our research focus should be on applied research aiming to have more innovation and added values to available medicines.

So, Jordan Journal of Pharmaceutical Sciences (JJPS) invites you all to contribute your research towards making medical solutions available and accessible worldwide.

Prof. Bashar Alkhalidi

Editorial Board

CONTENTS

Formulation Design and Characterization of Transdermal Films of Amlodipine Besylate for Enhancing Therapeutic Efficacy

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ABSTRACT

This study is designed to develop Hydroxypropyl Methylcellulose (HPMC) based polymeric films of amlodipine besylate to explore HPMC as a rate retardant polymer in transdermal drug delivery system. Films were formulated by the solvent casting method. Physicochemical parameters of the films were evaluated (film permeability, elongation, pH, drug content uniformity and release kinetics). Results found that the elongation of formulated films were about 3.33-13.33%, whereas surface pH varies from 6.82 to 6.97. Maximum permeability flux was found 0.384 gm cm^{-2}/day for ABF7; whereas minimum for ABF3 (0.338 gm cm^{-2}/day). After 8 hours, the highest release (95.77%) was for ABF6; whereas 83.67% (least) release was for ABF3 and ABF5. For dissolution patterns, among different kinetic models, the Higuchi model was fitted nicely for release kinetic of the formulated films. It revealed that the drug release pattern and kinetics were affected due to the change in the polymer-drug ratio and solvents. It showed that HPMC plays a significant role in dissolution rate. In conclusion: we found that ABF7 is the best fit formulation for preparing HPMC based transdermal film of amlodipine besylate. Further investigation is warranted to correlate *in-vitro* and *in-vivo* findings for the potential therapeutic use.

Keywords Amlodipine besylate, Transdermal film, HPMC, Dissolution, Release Kinetics.

1. INTRODUCTION

Transdermal drug delivery (TDD) is an acceptable mode of drug delivery for administering the drug into the body across the skin. TDD is not a new concept; for thousands of years, people use medication on the skin for healing local injuries.[1] However, it takes more focus recent era as a useful mode of drug delivery. It is becoming popular in medical practice day by day due to its potentiality of using the alternative of oral and injectable dosage forms. Several advantages are noticed for TDD preparations, such as- avoidance of first-pass metabolism, delivery of steady infusion of the drug for a prolonged time, reduction of side effects, easy application and removal of TDD films/patches, patient acceptance, etc.[2,3] Moreover, it is possible to minimize the potential

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hazards (side effects) of the drug by infusing TDD drug through skin rather than intravenous infusion (a superior mode of drug delivery).[4]

Hydroxypropyl methylcellulose (HPMC) is a semi-synthetic polymer that is inert in nature, and used to prepare controlled, sustained released, and other dosage forms (tablets, films, etc).[5,6] On the other hand, amlodipine besylate belongs to the group of medication 'dihydropyridine type Ca^{2+} blockers' used to lower blood pressure and chest pain. $t_{1/2}$ of this drug is 35-50 hours and bioavailability of almost 60-65%.[7]

There are few studies have been done on amlodipine besylate for preparing TDD films[8-10]; however, more information is demanding before using it clinically. To confirm the characteristics of an ideal TDD film of amlodipine besylate, we have formulated and later investigated the different parameters of the films like-permeability profile, release pattern, pH, elongation profile, content uniformity, etc. In fact, our goal is to prepare a suitable, easier, cost-effective, controlled release amlodipine-loaded transdermal film using HPMC polymer. The retarding effects of different formulations using HPMC and solvents of different ratios have also been compared in this study.

MATERIALS AND METHODS

Materials

Amlodipine besylate (Cadila Healthcare Ltd., India) was received from Glove Pharmaceuticals Ltd., Bangladesh. Methanol, ethanol, and chloroform are from (Merck, Germany); potassium dihydrogen phosphate (BDH, UK) was collected from the analytical laboratory of Dept. of Pharmacy, NSTU, Bangladesh. All other reagents employed were analytical grades.

Formulation of the films

The transdermal films of amlodipine besylate were formed by solvent casting technique according to Rita et al. with slight modification.[11] Films were prepared by different amounts of HPMC, drug, and solvent which are presented in Table 1. Precise weight of drug and HPMC were mixed properly; and then different solvents- methanol, ethanol, chloroform were poured at prerequisite amount into the mixtures in a 25 ml volumetric flask. The homogeneous mix was poured onto petridish (D-75mm) and then kept in dry place at room temperature for evaporating the solvents. The petridishes were lubricated using paraffin oil for easy removal of the films. Films were discharged from the incubated dish after 24 hours; and then kept in the desiccator for next use.

Table 1. Formulation of amlodipine besylate loaded films containing HPMC

Formulations	Amlodipine (mg)	HPMC (mg)	Solvent (ml)
ABF1	100	100	15 (methanol)
ABF2	100	200	15 (methanol)
ABF3	100	100	15 (chloroform)
ABF4	100	200	15 (chloroform)
ABF5	100	100	15 (ethanol)
ABF6	100	200	15 (ethanol)
ABF7	100	200	5 (methanol) + 5 (ethanol) + 5 (chloroform)

AB = Amlodipine besylate

Film permeability test

4 cm^2 (2x2 cm) areas of the film were cut down from different parts of the patch. Calcium chloride solution in water was taken on a glass bottle and the film was attached to the opening of the bottle. The weight of the $CaCl_2$ filled bottles were taken again. By subtracting the bottle weight, the weight of the $CaCl_2$ solution was determined. Daily weight losses due to permeability of the films were recorded for 10 days period.[12]

Percentage elongation test

Small strips were cut down from the center, left, and right side positions of each film. At first, the primal length was measured. Then, the sliced strips were pulled on both sides until breaking point. The percentage of the increment of length was measured by using the following equation.[11]

Percentage of elongation = $(l_2-l_1)/l_1$ x 100; (l_1= Initial length of strip; l_2 = Final length of strip)

Surface pH test

The pH of the formulated films was studied in this experiment. In this aspect, 4 cm^2 (2x2 cm) pieces from different locations of tested films were cut down and allowed to swell up by keeping in distilled water (in glass tube) for 1 hour. Surface pH was measured by using a glass electrode, which was allowed to bring nearer to the film surface for a 1 minute period (during this time, pH meter can equilibrate with the surface of the films).[13]

Uniformity of drug content study

Drug content was determined for all prepared films. 4 cm^2 strips of the films were cut and weigh accurately. Then these strips were chopped up and taken into a measuring flask. The dissolving solvents (5 ml) by which the film was formed was poured in it. Strips were solvated completely through handshaking. Distilled water was added slowly for adjusting the volume up to 10ml. The solution was filtered after mixing. The filtrate was diluted using distilled water, and the dilution factor was noted for calculation. Finally, the samples were measured spectrophotometrically at 360 nm[11] (λ_{max} of amlodipine besylate) by Shimadzu UV spectrophotometer for drug content.[14]

***In-vitro* dissolution study**

In-vitro dissolution studies of our prepared films were done according to Rita et al. with slight modification.[11] For preparing buffer (pH 7.4), 0.68 gm potassium dihydrogen phosphate was weighed properly and then dissolved in 1L distilled water. NaOH was added for adjusting the pH. We have used USP XXII dissolution apparatus II (paddle) for studying drug release pattern from films. 4 cm^2 (2x2 cm) slices of the dry transdermal films were cut down and fixed over the glass plates. As a result, the drug can only release from one side of the film. The glass plate was placed at the bottom of the dissolution tester, which contained 500 ml dissolution medium at 37°C ± 0.5°C. The distance between the paddle and glass plate was 2.5 cm. We also fixed the rotation at 50 rpm. Five ml aliquot was withdrawn at regular intervals for analysis, which was replaced by the fresh phosphate buffer of same amount. Collected samples were filtered and later analyzed by UV spectrophotometer at 360 nm for getting the amount of dissolved drug released from the films.[14,15]

Drug release kinetics

After taking the data of 8 hours dissolution study of the formulated films, we have used different kinetic models for finding the best fit model of each formulation. The kinetic models were listed below-

Zero-order release model[16]: $Q_t = Q_0 + K_0t$;(Q_t= Amount of drug release/dissolved, Q_0 = Amount of drug at initial time in solution, K= Zero-order rate constant)

First order release model[17]: $LnC = LnC_0 + K_1t$; (C_t = Concentration release/dissolved drug, C_0 = Concentration of drug at initial time, K_1= First order rate constant)

Higuchi model[18-20]: $M_t = M_0 + K_Ht^{1/2}$; (M_t = Amount of released drug (cumulative), M_0 = Amount of drug at initial time, K_H = Higuchi release constant)

Korsmeyer-Peppas model[20-23]: $M_t/M_\infty = Kt^n$; (M_t/M_∞ = Drug release fraction at time t, K= Release rate constant). Here, 'n' is the release exponent, and this value is used to characterize different releases from the cylindrical shaped matrix. During drug release from a cylinder-shaped matrix, the release exponent value $n \leq 0.45$ demonstrates the Fickian diffusion mechanism. That means the drug may release from the matrix due to erosion of the polymeric chain.

On the other hand, the value of 'n' in the Korsmeyer-Peppas equation ($0.45<n<0.89$) refers to a non-Fickian (anomalous) transport mechanism. It refers that drug release is the combination of both diffusion and erosion controlled.[21]

Statistical analysis of data

Descriptive statistical analysis was done in this study by applying MS Excel 2007; and values are presented as (mean ± SD).

RESULTS AND DISCUSSION

Evaluation of film permeability studies

Permeation of $CaCl_2$ solution from the container (glass bottle) through the films was studied for 10 days. Gradual weight loss of the $CaCl_2$ solution confirmed the permeability of formulated films (Table 2). In Table 3, we found that the permeation flux value was maximum for ABF6 and ABF7, which was 0.384gmcm^{-2}/day. This indicated better permeation of films than others. Permeation coefficient indicates the rate of permeation (weight loss of solution) through the films. Maximum permeability coefficient (K_p) was found for ABF5, whereas least was observed for ABF6. Permeability co-efficient for ABF1, ABF2, ABF3, ABF4, ABF5, ABF6 and ABF7 were found as 0.081, 0.078, 0.043, 0.075, 0.124, 0.018 and 0.048 respectively.

Table 2. Comparison of film permeability among the prepared transdermal films

Formulation	Weight of container (gm)	Weight of container + $CaCl_2$ solutions (gm)	Net weight of $CaCl_2$ solution (gm)	Weight loss due to permeability of film (gm)					Weight loss of $CaCl_2$ solution (%)
				1 day later	2 days later	3 days later	7 days later	10 days later	
ABF1	69.375	124.732	55.375	54.305	53.532	52.650	51.732	47.352	14.48
ABF2	61.234	117.543	56.309	55.549	54.501	53.751	52.425	48.459	13.94
ABF3	67.572	122.356	54.784	53.652	52.702	51.549	50.135	47.356	13.50
ABF4	61.675	121.572	59.897	58.325	57.634	56.754	55.576	51.269	14.40
ABF5	65.432	116.378	50.946	50.240	49.576	50.575	47.765	43.256	15.09
ABF6	60.354	112.276	51.922	50.546	49.254	48.203	45.305	43.958	15.35
ABF7	54.935	105.461	50.526	49.725	48.375	47.298	45.509	42.759	15.37

Table 3. Permeation flux and permeability co-efficient study of transdermal films

Formulation	Permeation flux (gmcm^{-2}/day)	Permeability co-efficient (Kp)
ABF1	0.362	0.081
ABF2	0.349	0.078
ABF3	0.336	0.043
ABF4	0.360	0.075
ABF5	0.377	0.124
ABF6	0.384	0.018
ABF7	0.384	0.048

Evaluation of percentage elongation, surface pH, and content uniformity of the films

Elongation of the films can be defined as the increment of sample's gauge length just before the break point divided by the sample's original length. The greater the elongation, the higher the ductility or elasticity of the

materials. On the other hand, surface pH gave us information for predicting the formulated films are either safe or irritating for the skin. Content uniformity tests were performed to confirm the homogenous distribution of the drug (amlodipine besylate) throughout the whole film.

Comparisons of these three parameters of different films are shown in Table 4. The elongation percentage of the films varied from (3.33 ± 0.36) to (13.33 ± 0.35) %. ABF3 showed the highest percentage of elongation than other formulations, which means this film has good ductility and elasticity. Due to This value is satisfactory also for the other formulations also. Surface pH was measured for each formulation, and it was noticed that the pH of all formulations was very near to the neutral pH (□7). The overall pH of the human body is neutral (pH = 7). Although the skin's pH is slightly acidic in some cases, human skin can be easily exposed to neutral pH. The pH range of our formulated film is (6.82-6.97), which confirms the safety and non-irritation profile of the formulations. The content uniformity test confirmed that the distribution of the drug among the films was homogenous. We took strips from the different portions of the films, and then drug content was measured. It was found that the drug content (in 4 cm^2 strip) of the films of different formulations is (1.24 ± 0.008) mg/ml (mean ± STD). The deviation of content uniformity among the films is very little; hence it can be said that the results are satisfactory.

Table 4. Physicochemical evaluation of transdermal films

Formulation	Elongation (%) (mm)	Surface pH	Content uniformity/4 cm^2 film (mg/ml)
ABF1	6.67 ± 0.45	6.94 ± 0.12	1.24 ± 0.19
ABF2	10.0 ± 0.33	6.85 ± 0.15	1.38 ± 0.09
ABF3	13.33 ± 0.35	6.82 ± 0.13	1.25 ± 0.14
ABF4	10.0 ± 0.45	6.97 ± 0.16	1.13 ± 0.06
ABF5	3.33 ± 0.36	6.87 ± 0.20	1.23 ± 0.12
ABF6	6.67 ± 0.42	6.93 ± 0.23	1.18 ± 0.09
ABF7	6.67 ± 0.25	6.89 ± 0.14	1.25 ± 0.10

Value = Mean ± SD, n=3

Evaluation of *in-vitro* dissolution pattern from the films

The dissolution studies of the formulated films ABF1, ABF2, ABF3, ABF4, ABF5, ABF6, and ABF7 were carried out as USP paddle method. We have recorded the release profile of the drug for 8 hours period. (Figure 1) and (Figure 2) display Zero-order and Higuchi plots of release rate of amlodipine besylate from the films, respectively. After 8 hours, the overall drug release from the formulated films- ABF1, ABF2, ABF3, ABF4, ABF5, ABF6, ABF7 were 89.7%, 91.2%, 87%, 89.3%, 87.0%, 95.77%, and 93.1%, respectively. It was noticed that our formulated films could retard the drug release from the films for up to 8 hours. Drug release was gradual in most of the cases, and the release range from the films was (87-95.77)% after 8 hours of the study.

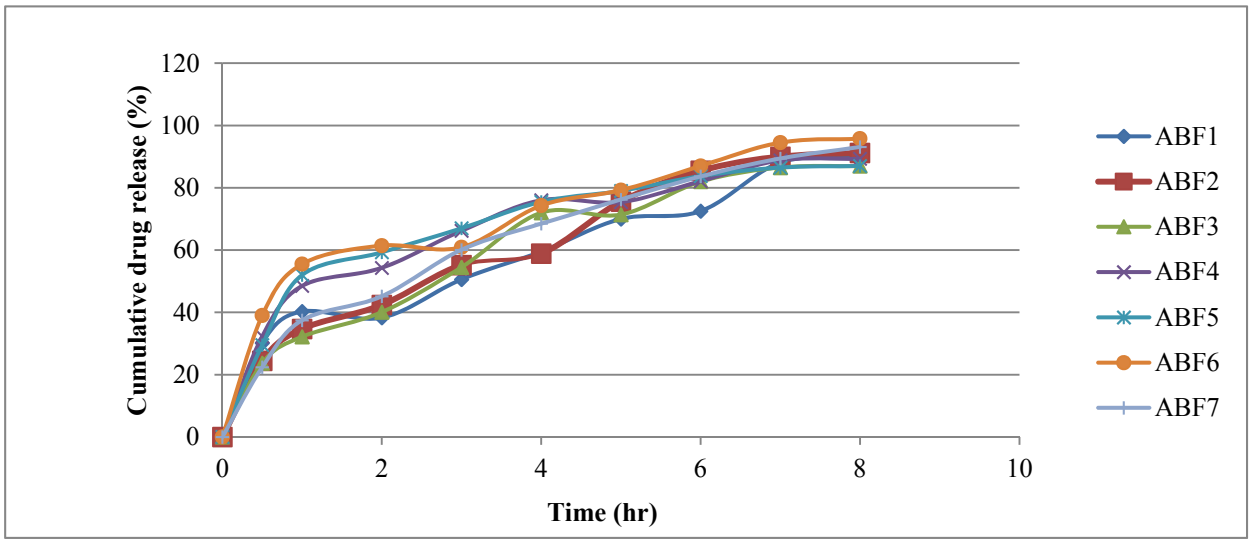

Figure (1): Zero order release kinetics of amlodipine besylate from films

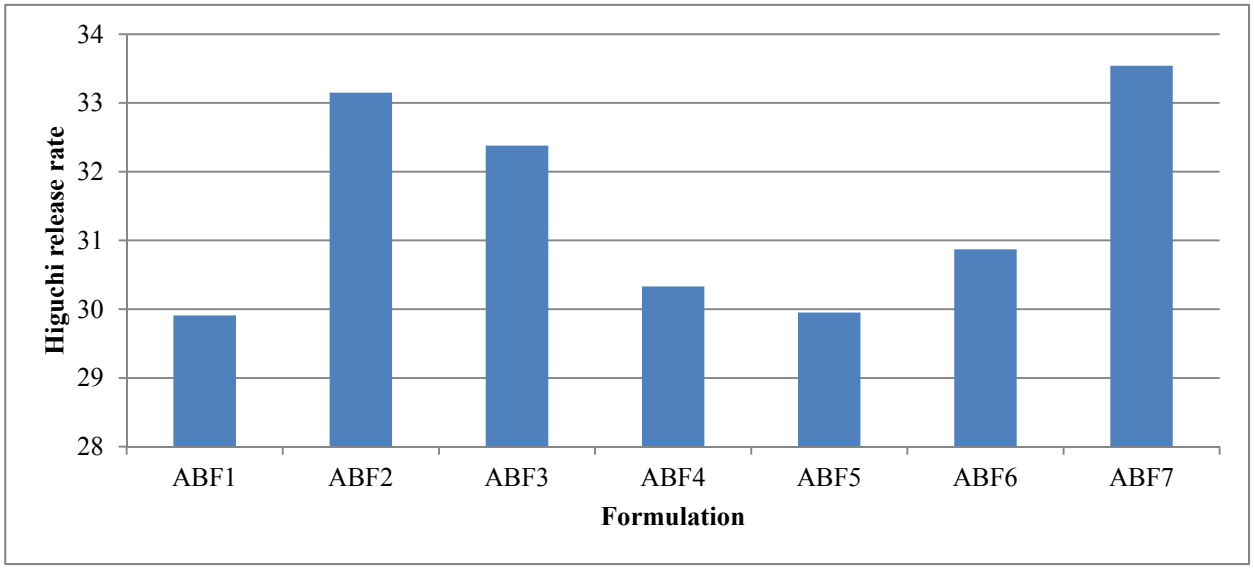

Figure (2): Higuchi release rate of amlodipine besylate from films

Table 5 represents the kinetics data of the formulated transdermal films. ABF1 best fit with Higuchi (R^2 = 0.967) kinetic model. Similarly ABF2, ABF3, ABF6, and ABF7 followed Higuchi kinetic model where R^2 values are 0.986, 0.985, 0.947, and 0.996, respectively. The rates of Higuchi release from films are shown in (Figure 2). ABF4 and ABF5 displayed Korsmeyer kinetic model with R^2 values of 0.971 and 0.988, respectively.

Release exponent value (n) of Korsmeyer release model for the formulations of ABF1, ABF2, ABF3, ABF4, ABF5, ABF6, ABF7 were 0.440, 0.509, 0.524, 0.314, 0.266, 0.281, and 0.465, respectively.

Table 5. Release kinetics of amlodipine besylate from different films

Formulation	Zero order		First order		Higuchi		Korsmeyer-Peppas	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	n
ABF1	0.911	9.475	0.931	-0.111	0.967	29.91	0.885	0.440
ABF2	0.932	10.510	0.969	-0.132	0.986	33.15	0.968	0.509
ABF3	0.909	10.150	0.980	-0.113	0.985	32.38	0.968	0.524
ABF4	0.809	9.072	0.967	-0.113	0.963	30.33	0.971	0.314
ABF5	0.766	8.813	0.950	-0.105	0.942	29.95	0.988	0.266
ABF6	0.805	9.288	0.941	-0.152	0.947	30.87	0.932	0.281
ABF7	0.913	10.480	0.986	-0.135	0.996	33.54	0.985	0.465

To confirm the drug release pattern, 'n' value was determined from Korsmeyer-Peppas equation. It was found that release exponent 'n' was ≤0.45 for the formulations ABF1, ABF4, ABF5, and ABF6. That means drug release from the matrix of these films was Fickian diffusion mediated. On the other hand, formulations ABF2, ABF3, and ABF7 follow anomalous diffusion/non- Fickian as the value of n was ($0.45 < n < 0.89$). This indicates that drug releases from these films were done through both diffusion and erosion controlled mechanism.[19-21] It was observed that Korsmeyer-Peppas kinetic represents good linearity for the formulations ABF5 and ABF7 (R^2 value is very much nearer to 1). However, in the Higuchi model R^2 value is the closest to 1 for the formulation ABF7. Hence, it can be said that ABF7 is the best fit model among our studied formulations.

CONCLUSION

The present study reveals that all the physical parameters for ideal film formation were satisfactory for the manufacturing process. We found that our formulated films can retard drug release up to 8 hours, and in most cases, the drug release patterns follow the anomalous/non-Fickian transport process. Among the experimental formulations of our study, ABF7 is the best fit formulation for preparing transdermal films. It is also possible to modulate the rate and extent of drug release from prepared films, which might be useful for preparing the desired formulation by the judicious addition of drug and polymer (HPMC). Further studies are recommended to correlate the *in-vivo* and *in-vitro* experimental findings to confirm the accurate pattern of drug release before using the films therapeutically.

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تصميم وتوصيف الأغشية عبر الجلد لأملوديبين بيزيلات لتعزيز الفعالية العلاجية

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ملخص

صممت هذه الدراسة لتطوير أغشية بوليمرية أساسها هيدروكسي بروبيل ميثيل سلولوز (HPMC) من أملوديبين بيسيلات لاستكشاف HPMC كبوليمر مثبط للمعدل في نظام توصيل الأدوية عبر الجلد. تمت صياغة الأغشية بطريقة الصب بالمذيبات.تم تقييم المعلمات الفيزيائية والكيميائية للأغشية (نفاذية الفيلم ، الاستطالة ، الأس الهيدروجيني ، توحيد محتوى الدواء وحركية الإطلاق).ووجدت النتائج أن استطالة الأغشية المركبة كانت حوالي 3.33−13.33٪ ، بينما تراوح الأس الهيدروجيني السطحي من 6.82 إلى 6.97. تم العثور على أقصى تدفق للنفاذية 0.384 جم / سم 2 / يوم لـ ABF7 ؛ بينما الحد الأدنى لـ 0.338) ABF3 جم سم −2 / يوم). بعد 8 ساعات ، كان أعلى إطلاق (95.77٪) لـ ABF6 ؛ بينما كان الإصدار 83.67٪ (الأقل) من أجل ABF3 و ABF5. بالنسبة لنمط الذوبان ، من بين النماذج الحركية المختلفة ، تم تركيب نموذج Higuchi بشكل جيد للإفراج عن الحركة الحركية للأغشية المركبة. وكشفت أن نمط إطلاق الدواء والحركية قد تأثروا بسبب التغير في نسبة البوليمر إلى الدواء والمذيبات. أظهر أن HPMC يلعب دورًا مهمًا في معدل الذوبان. في الختام ، وجدنا أن ABF7 هي أفضل صيغة مناسبة لإعداد فيلم عبر الجلد يعتمد على HPMC من أملوديبين بيسيلات. هناك ما يبرر إجراء مزيد من التحقيق لربط النتائج في المختبر وداخل الجسم من أجل الاستخدام العلاجي المحتمل.

الكلمات الدالة: أملوديبين بيسيلات ، فيلم عبر الجلد ، HPMC ، الذوبان ، الخواص الحركية.

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The Efficacy and Safety of Metformin and Glimepiride Combination among Jordanian Patients with Type 2 Diabetes during Ramadan

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ABSTRACT

The Aims of the study: The prevalence of Type 2 diabetes mellitus (T2DM) is increasing worldwide including Islamic countries. Ramadan fasting increases risk of complications from T2DM especially hypoglycemia. The primary objective of the study was to assess glycemic control and incidence of hypoglycemia before, during and after Ramadan. Methods: One hundred adult Jordanian patients with Patients with T2DM who were on dual therapy with metformin and glimepiride and practice Ramadan were recruited. Glycemic control was assessed by HbA1c and fasting blood glucose (FBG), in addition to number of hypoglycemic episodes, before, during and after Ramadan. Results There was a significant decrease in HbA1cand FBG at the end of Ramadan compared to that before Ramadan (10.6±1.9 vs. 9.4±1.7 and 386.4±110) vs. 271.9 ±102.4; respectively). On the other hand, the number of hypoglycemic episodes was significantly less six to eight weeks after Ramadan than that during Ramadan ($p<0.001$), and those during Ramadan were significantly less than those prior to Ramadan. No statistically significant correlation ($p>0.05$) was detected between the number of hypoglycemic episodes and FBG or HbA1c at all visits. However, the percentage of patients with hypoglycemia during Ramadan was higher than that of after Ramadan (86% vs. 41%, $p<0.01$). Conclusion: While Ramadan fasting might be considered efficacious in patients with Patients with T2DM on combination of glimepiride and metformin, patients and/or their caregivers should be educated on the monitoring for signs and symptoms of hypoglycemia.

Keywords Hypoglycemia, Glimepiride, Metformin, Ramadan, Type 2 diabetes.

1. INTRODUCTION

Diabetes mellitus is a widespread chronic progressive disease with a global prevalence that is worryingly growing. In 2013,382 million people suffered from diabetes and the number is estimated to rise to 592 million by 2035[1].Because of the urbanization and socioeconomic developments, a 10% annual increase in the prevalence of diabetes was observed in countries with large Muslim populations[2]. Worldwide, almost 1.6 billion of the world's population follows Islam.

Fasting during the month of Ramadan is essential to Islamic faith. Ramadan is the ninth month of the Islamic lunar calendar. During this month, Adult Muslims are required to abstain from eating, drinking, smoking and taking oral drugs between sunrise and sunset[3].Ramadan can occur any time in the year and lasts for up to 30 days. The duration of fasting can range from a few to more than 20 hours depending on the geographic location and the

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season of the year. Islam exempts people with serious illness from the obligation of fasting, if fasting might adversely affect patient's condition. Although Patients with diabetes fall in this category, many diabetic patients insist to fast the holy month of Ramadan[4]. The population-based Epidemiology of Diabetes and Ramadan study (EPIDIAR) showed that almost 43%of patients with Type 1 diabetes(T1DM) and 79%of patients with Type 2 diabetes (T2DM) from 13 Islamic countries reported fasting for at least15 days during Ramadan[5]. Ramadan Fasting increases the potential complications in patients with T2DM. The EPIDIAR study also showed that fasting increased significantly the risk of severe hyperglycemia by 5-fold and severe hypoglycemia by 7.5-fold in diabetic patients during Ramadan, compared with previous months. Moreover, up to 2% of the fasting Patients with T2DM required hospitalization because they experienced at least one episode of severe hypoglycemia [5], [6]. Therefore, in the context of fasting, healthcare professionals need to be considerate to patients who are eager to fast [7]. If modifications can be made to a patient's treatment regime, they can be counselled about these. Pharmacists plays an important role as they can offer professional advice on the management of medicines and can communicate with the patient's endocrinologist to make any necessary changes to their medicines regime in preparation for Ramadan.[8]

The primary objective of the study was to assess glycemic control before, during and after Ramadan in terms of HbA1c and FBG. The glycemic control between newly diagnosed subjects and subjects already being treated for T2DMat the end of the study was assessed.

The secondary objective of the study was to assess the incidence of hypoglycemic episodes before, during and after Ramadan. The relationship between glycemic control and number of hypoglycemic episodes before and during Ramadan was also evaluated.

Research design and Methods

This was an observational study of Muslim patients with T2DMreceivingdual therapy of metformin and glimepiride during the holy month of Ramadan. Patients who aged 18 years and over, and willing to participate in fasting throughout Ramadan month, were included in the study. Participants attended diabetes clinic in Prince Ali University Hospital and Jordanian Royal Medical Services. The study was carried out From June to September in 2017. All patients participating in the study received an education regarding the management of their diabetes and identification of hypoglycemia events during Ramadan.

Sample size was done by G*power 3.1.10 base on study by Shin *et al.*[9], that is to detect a difference of 1 unit in HbA1c before and after Ramadan, and have 80% power, and 0.05 error, a 89 patients would need to be recruited. Therefore, we recruited 100 patients to account for dropouts.

Accordingly, out of 143 patients approached, a total of 100 Patients with T2DM treated with dual therapy of metformin-glimepiride who practiced Ramadan fasting were included to the study (response rate 70%). Fifty patients were newly diagnosed subjects (diagnosed in the 12 months or less preceding inclusion) and the remaining were subjects already being treated forT2DM.

Patients were requested to attend diabetes clinic in four visits. A screening visit (Visit 0) was at the inclusion of the study (One month before Ramadan). Visit 1 was just at the start of Ramadan (Within the first five days of Ramadan). Visit 2 was at the end of Ramadan (Last five days of Ramadan). Finally visit 3 was between 45-60 days after the end of Ramadan. At every visit, the number of hypoglycemic episodes and a routine laboratory tests including fasting blood glucose (FBG) and glycosylated hemoglobin (HbA1c) were assessed. Hypoglycemic episodes were defined asymptomatic; based on self-monitoring of blood glucose using a glucometer (defined as ≤70 mg/dL).

During there- and post-Ramadan observation periods, subjects administered the study drugs as usually prescribed. During Ramadan, study drugs were

administered at the time of breaking the fast in the evening. The doses of study drugs were kept stable throughout the length of the study, except when medically contraindicated (recurrent hypoglycemia or hyperglycemia reaching 250 mg/dl or more).

The following patients were not eligible for this study: patients with type 1 diabetes, patients with T2DM treated with insulin or blood glucose-lowering agents other than the study medication, pregnant or breastfeeding women, patients with known hypersensitivity to metformin or glimepiride, and finally patients with diabetic ketoacidosis or/and progressive fatal disease.

Statistical analysis

Mean and standard deviation were used to describe continuous variables, and percentages to describe categorical variables. Repeated-measures ANOVA was used to compare glycemic control at V0, V1, V2 and V3 followed by Tukey's post-hoc analysis. Independent t-test was used to compare glycemic control in patients with short vs. long duration T2DM. Number of hypoglycemic episodes at V0, V1, V2 and V3 was compared using Wilcoxon-Signed Ranks test followed by post-hoc analysis. Percentage of patients with hypoglycemia episodes before, during and after Ramadan was compared using McNamara test between each of the two groups. Spearman correlation was done to assess the relationship between HbA1c, FBG and number of hypoglycemic episodes. All statistical tests were considered significant at p-value less than 0.05. SPSS® statistical software version20 was used to carry on all analyses.

Results:

Baseline characteristics

One hundred patients with T2DM were recruited. Table 1 describes baseline characteristics (demographics) of these patients.

Table 1: Baseline characteristics (demographics) of patients.

	Mean (SD)	Range
Age (year)	57.4 (13.2)	(30-93)
Weight (kg)	77.4 (11.3)	(55-105)
Height (cm)	169.2 (7.1)	(155-190)
BMI (kg/m^2)	26.8 (2.8)	(23-29)
Time since first diagnosis (years)	26.5 (24.4)	(2-120)
With 12 months	50%	
More than 12 months	50%	
Glimepiride daily dose (mg)	5.9 (35)	(2-16)
Gender		
Female (%)	56	
Male (%)	44	

Glycemic control (as measured by HbA1c and FBG)

Repeated-measures ANOVA was used to compare HbA1c and FBG at V0, V1, V2 and V3 as depicted in **table 2**. The mean HbA1c values were statistically significantly different among visits [F (1.7, 167.7) = 106.5, p < 0.0001]. Mean FBG levels were similarly statistically significantly different [F (2.113, 209.231=77.06, p<0.0001]. Post-hoc Bonferroni correction revealed that there was a significant drop in HbA1c after the beginning, during (V0 vs. V1, V2, V3 and V3, V1 vs. V2 and V3; p<0.0001) and after Ramadan (V2 vs. V3, p=0.001). As for FBG levels, there was a

statistically significant decrease after the beginning, during (V0 vs. V1, V2, and V3, V1 vs. V2 and V3; p<0.0001) but **not** after Ramadan (V2 vs. V3, p=1.00).

Table 2: Glycemic control before, during, at the end and after Ramadan. Data are represented as means.

Parameter Mean (SD)	Visit 0	Visit 1	Visit 2	Visit 3
HbA1c (%)	10.2 (1.9)	9.8 (1.8)	9.4 (1.7)*	9.2 (1.5)
FBG (mg/ld.)	386.4 (110)	325.5 (97.8)	271.9 (102.4)*	258.3 (100.5)

*P<0.05 for visit 2 vs. visit 0.

Glycemic control (according to duration of diabetes)

HbA1c and FBG were compared at each time point between patients which were diagnosed within 1 year and those diagnosed for more than one year (Table 3). Although, HbA1c values tend to be higher in those with longer time since diagnosis, no statistically significant differences were detected between both HbA1c and FBG at V3, where FBG was significantly higher in patients with longer duration of disease (after Ramadan).

Table 3: Glycemic control according to duration of diabetes at each time point. Data are represented by means (*p<0.05).

Parameter	Time since diagnosis	
	Within 12 months (n=50)	More than 12 months (n=50)
HbA1c- visit 0	9.9 (1.9)	10.4 (1.9)
FBG- visit 0	391.9 (117.3)	380.9 (103.0)
HbA1c-visit 1	9.6 (1.7)	10.0 (1.8)
FBG (mg/dl)- visit 1	330.5 (107.2)	320.6 (88.1)
HbA1c-visit 2	9.2 (1.7)	9.5 (1.6)
FBG (mg/dl)- visit 2	265.2 (93.2)	278.5 (111.3)
HbA1c-visit 3	9.0 (1.6)	9.4 (1.5)
FBG (mg/dl)- visit 3	238.3 (95.4)	278.3 (102.3)*

Frequency of hypoglycemic episodes

The number of hypoglycemic episodes was significantly less post-Ramadan than that during Ramadan (p<0.001), and those during Ramadan were significantly less than those prior to Ramadan. No statistically significant correlation (p>0.05) was detected between the number of hypoglycemic episodes and FBG or HbA1c at all visits (data now shown).

In addition, the percentage of patients with and without hypoglycemia was compared before, during and after Ramadan (Table 4). Percentage of patients with hypoglycemia was significantly higher before and during Ramadan than those six to eight weeks after it.

Table 4: Number of hypoglycemic episodes before, during, and after Ramadan. Described as median and Inter-quartile range (IQR).

Time point	Median (IQR)	Patients with hypoglycemia (%)
Visit 0	2 (0-4)	71
Visit 1	1 (1-2)	86
Visit 4	0 (0-1)	41*

*P<0.01 for visit 4 vs. visit 1.

Discussion

Fasting is optional during the holy month of Ramadan for diabetic patients. Yet, many Muslims prefer to abstain from food and water for about 12-20 hours in observance of this pillar. This might expose these patient to less glycemic control and higher risk of hypoglycemia when compared to that of non-fasting periods, especially when taking medications that can increase the risk of hypoglycemia , namely insulin and sulfonylureas[10]. The aim of the present study was to compare glycemic control, and hypoglycemia in Patients with T2DM_ who are treated with a combination of metformin and glimepiride_ before, during and 6 to 8 weeks after Ramadan. Our results showed that Ramadan fasting did not affect the efficacy nor safety of the combination from glimepiride and metformin.

Previous literature showed similar findings to our study. M'guil and colleagues (2008) [11] assessed the safety of Ramadan fasting in 120 Moroccan patients (62 females, 58 males) with T2DM who were on diet and/or gliclazide (belongs to sulfonylureas). Our study, however, evaluated the effect of a combination of metformin and a sulfonylurea (glimepiride). Various parameters were measured at four occasions. Prior Ramadan fasting, days 15 and 29 days of fasting and 15 days after Ramadan ends. With regard to diabetes control, only in females, fasting and 2-hr postprandial glucose levels, and insulin-like growth factor (IGF-1) decreased during Ramadan but returned to be close to baseline values after Ramadan was over. The later results resembles our finding as patients had hypoglycemia during Ramadan, but recovered during the post-Ramadan period. Insulin levels exhibited the same later pattern in both males and females. HOMA-IR continued to decrease even when assessed 15 days after fasting. Plasma fructosamine decreased after fasting in males, but plasma C-protein and HbA1c did not change, like our findings

Similarly, Sahin *et al.* (2013) [9], observed 122 patients with T2DM (67.2% females) to evaluate their glycemic control during Ramadan in fasting and non-fasting group. Patients were treated with different antidiabetic regimens. About 66% of patients were treated with mono- or biotherapy of oral antidiabetic drugs (OAD), 7.4 % with a combination of OAD plus eventide, 6.5 % with a combination of insulin plus OAD divided between glandes and metformin or sit gliptin),and 19.7 % with insulin alone. These percentages were similar in both arms of the study. Comparable to our findings, Shin *et al.* showed that there was a tendency of higher frequency of hyperglycemia and hypoglycemia episodes during fasting but this was not statistically significant. However, the frequency of hyperglycemia was associated with reduction of insulin dose. In our study, we did not have insulin, and the dose of both medications was constant throughout the study which gives our results more conformity. Moreover, other parameters of glycemic control including fasting blood glucose (FBG), post-prandial glucose (PPG), fructosamine, HbA1c and fasting insulin did not change significantly in the fasting group. In Turkey, the effects of glimepiride (n=21), repaglinide (n=18), and insulin glargine (n=10) in fasting Patients with T2DM on the

glucose metabolism were compared to non-fasting controls [12]. FBG, PPG, HbA1c, and fructosamine were assessed in before Ramadan, immediately after Ramadan and 1-month after Ramadan. There was no significant change in FBG, PPG, and HbA1c variables in fasting diabetics before Ramadan when compared to those shortly after Ramadan and 1-month after Ramadan. The later matched results from our study. However, PPG was found to be significantly higher in***n on-fasting*** control diabetic sat both time-points after Ramadan. Fructosamine levels increased significantly in both fasting group and non-fasting group 1-month after Ramadan in patients treated with glimepiride or repglinide or glargine. Risk of hypoglycemia did not significantly differ between fasting and non-fasting diabetics in patients treated with three drug therapies [12]. However, the later study did not evaluated the combined effect of metformin and glimepiride. The glimepiride study group (GSG) (2005)[13] evaluated the effect of Ramadan fasting on control of T2DM in 332 patients, form 33 centers in six countries (Algeria, Egypt, Indonesia, Jordan, Lebanon, and Malaysia), controlled on glimepiride ***only***, unlike ours which evaluated a biotherapy with metformin One hundred of patients were newly diagnosed and 232 were already-treated. Patients were assessed at baseline (V0 or inclusion visit), start (V1), during (V2) and 45-75 day after Ramadan (V3). HbA1c values decreased significantly in both newly diagnosed and subjects who were already treated. The mean FBG value at the baseline, simultaneously, decreased. In line with our findings, hypoglycemic events were 25 at V1, 15 at V2 and 8 at V3, especially in the already treated group. GSG concluded that the efficacy and safety of the same median dose of glimepiride (2 mg/day) in Patients with T2DM was not changed during Ramadan fasting, even when the time of administration of glimepiride is changed from the morning to the evening.

In continuum to the work of GSG[13], the significance of our study is that it was first relatively large observational longitudinal study to evaluate the potential effect of Ramadan fasting on glycemic control and hypoglycemia in Jordanian patients with T2DM taking a combination of metformin and glimepiride. The efficacy of this combination was unaltered in our 100 patients. ***However,*** the incidence of ***hypoglycemia was lower after Ramadan*** when compared to that of during and pre-Ramadan time points. But, no symptomatic or server hypoglycemic episodes were reported. Our study is limited by the lack of non-fasting control Patients with T2DM although patients' baseline can serve as their own controls. Future studies should aim to compare fasting patients to non-fasting counterparts. Also, since patients were visiting outpatient clinics, their adherence to lifestyle recommendations and medication could not be accurately assessed.

Conclusion

While Ramadan fasting might be considered efficacious in Patients with T2DM on combination of glimepiride and metformin, patients who insist on fasting and/or their caregivers should be assessed before and during Ramadan, and educated on their dietary plans, physical activities, as well as monitoring for signs and symptoms of hypoglycemia.

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Compliance with ethical standards

Conflict of interest the authors declare that they have no conflict of interest.

Ethical standard Approval for the study was obtained from the ethical committee associated with the Faculty of Medicine at Muta University and Royal Medical Services (Reference number: 20170).

Human rights all procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Informed consent Informed consent was obtained from all patients for being included in the study.

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فعالية ومأمونية استخدام دواء محتوي على الميتفورمين وجليمبيريد في مرضى السكري من النوع 2 في الأردن خلال شهر رمضان

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ملخص

الأهداف: انتشار داء السكري من النوع 2 (T2DM) في جميع أنحاء العالم بما في ذلك البلدان الإسلامية في زيادة مستمرة. يزيد صيام رمضان من خطر حدوث مضاعفات من T2DM وخاصة نقص السكر في الدم. كان الهدف الأساسي من الدراسة هو تقييم التحكم في نسبة السكر في الدم ومعدل حدوث نقص السكر في الدم قبل وأثناء وبعد شهر رمضان. منهجية البحث: تضمنت الدراسة مائة مريض أردني بالغ من مرضى T2DM والذين كانوا يخضعون للعلاج المزدوج بالميتفورمين والجليميبيريد خلال الصيام. تم تقييم التحكم في نسبة السكر في الدم بواسطة HbA1c ونسبة الجلوكوز في الدم (FBG) ، بالإضافة إلى عدد نوبات سكر الدم ، قبل وأثناء وبعد شهر رمضان. النتائج: كان هناك انخفاض ملحوظ في HbA1c و FBG في نهاية شهر رمضان مقارنةً به قبل شهر رمضان (10.6 ± 1.9 مقابل 9.4 ± 1.7 و 386.4 ± 110 مقابل 271.9 ± 102.4, على التوالي). على الجانب الآخر ، كان عدد نوبات نقص السكر في الدم أقل بكثير من ستة إلى ثمانية أسابيع بعد رمضان مقارنة بشهر رمضان ($p<0.001$) ، وكانت تلك خلال شهر رمضان أقل بكثير من تلك التي سبقت رمضان. لم يتم الكشف عن ارتباط ذي دلالة إحصائية ($p>0.05$) بين عدد نوبات سكر الدم و FBG أو HbA1c في جميع الزيارات. ومع ذلك ، كانت نسبة المرضى الذين يعانون من نقص السكر في الدم خلال شهر رمضان أعلى مما كانت عليه بعد رمضان (86٪ مقابل 41٪ ،$p<0.01$). الاستنتاجات: في حين أن صيام رمضان يمكن اعتباره فعالًا في المرضى الذين يعانون من T2DM على مزيج من جليمبيريد والميتفورمين ، يجب تثقيف المرضى و / أو مقدمي الرعاية حول مراقبة علامات وأعراض نقص السكر في الدم.

الكلمات الدالة: نقص السكر فى الدم ، جليمبيريد ، ميتفورمين، رمضان ، السكري من النوع 2.

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Familiarity and Attitude toward Pharmacovigilance among Pharmacy Academics in Jordanian Universities: A Cross-Sectional Study

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ABSTRACT

Background: Awareness of pharmacovigilance among pharmacy academics increases the students and graduated pharmacists' knowledge and skills in reporting adverse drug reactions (ADRs).

Purpose: The aims of this study are to assess the level of awareness about pharmacovigilance among pharmacy school academics in Jordan and to explore the current pharmacovigilance education, from the academics' point of view.

Method: A paper-based 24-item questionnaire was distributed in eight schools of pharmacy that accepted to participate.

Results: The questionnaire was completed by 87 pharmacy academics. Most participants were familiar with the concept of pharmacovigilance (79.3%) and the majority (96.5%) agreed to implement ADR reporting in the school curriculum. Despite this positive attitude, only 26.4% of participants had previously attended a workshop about pharmacovigilance and 36.8% were able to educate the students on how to report ADRs.

Conclusion: Pharmacy school academics in Jordan have a good familiarity and positive attitude about pharmacovigilance. However, the inclusion of topics about detecting, preventing, and reporting ADRs to the current school curricula is recommended.

Keywords: Adverse drug reactions, Pharmacovigilance, Pharmacy education.

1. INTRODUCTION

The safe use of medicines is a high requisite in the healthcare world, so the science of pharmacovigilance has emerged [1]. The World Health Organization defines pharmacovigilance as "*the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug related problems*" [2]. Since adverse drug reactions (ADRs) are well known to cause morbidity and mortality or are associated with a lack of efficacy, overdose, abuse, or misuse, the reporting of ADRs is the cornerstone of pharmacovigilance activity. Studies have shown that ADRs are responsible for large numbers of hospital admissions [3-6]. In the United States, more than 100,000 annual deaths are attributed to serious adverse drug reactions [6]. In the UK, about 6.5% of hospital admissions are reported to be due to an ADR, and the overall mortality was 0.15% [4]. On the other hand, underreporting of ADRs is a real problem within pharmacovigilance. For instance, only 6% of all drug reactions are thought to be reported [7]; the main reason for underreporting is "not knowing" how to report [7,8].

Only one study assessed pharmacists' knowledge and attitudes toward ADR reporting in Jordan [9]. However, the results suggested that pharmacists have insufficient knowledge about pharmacovigilance and the reporting of

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ADRs. In addition, the study concluded that educational programs in the reporting process are necessary.

It is known that the level of knowledge regarding ADR reporting might be a significant factor that affects pharmacists reporting ADRs. Studies have demonstrated how education in pharmacovigilance can enhance ADR reporting. Of particular interest is a Danish study, which showed how through adequate training, pharmacy students can greatly improve the detection and reporting of ADRs by pharmacists [10].

Numerous studies have been done to evaluate the knowledge about ADR reporting and pharmacovigilance among students in different countries. These studies showed that the students have insufficient knowledge about ADR reporting and pharmacovigilance [10-14]. However, the awareness of pharmacovigilance among pharmacy academics can increase the students' knowledge and skills in reporting ADRs, but whether this observation has been captured within pharmacy academics was not investigated in Jordan. Studies have focused on the practicing pharmacists within the community as well as students, but not the academics. Therefore, the aims of this study are to assess the level of awareness about pharmacovigilance among pharmacy school academics and to explore the level of pharmacovigilance education provided to undergraduate pharmacy students in Jordan from the point of view of academics.

Method

A cross-sectional survey was conducted to obtain data relating to the familiarity, awareness, and attitudes toward pharmacovigilance at Jordanian schools of pharmacy between February 2017 and March 2017 in the second term of the academic year 2016-2017. The ethical approval to conduct the present study was obtained from Isra University (1/11/16).

The questionnaire was written in the English language (the teaching language at Jordan Universities) and was designed by the authors after extensive literature review on pharmacovigilance education. The draft questionnaire was tested for its face and content validity. Three independent members from the school of pharmacy assessed the relevance and clarity of the questionnaire and the ease of reading and understanding. The final version of the questionnaire was piloted among ten responses prior to launching.

The questionnaire consisted of 24 questions divided into three sections. The first section collected the demographic data of the respondents. The second section consisted of six questions and evaluated the respondent's familiarity with the definition of pharmacovigilance and policy in Jordan. The answer of each question was either yes or no, and a yes answer represented a familiarity with all questions. The last section explored the attitude toward pharmacovigilance teaching. It consisted of 5-item Likert scale questions from 1 (strongly disagree) to 5 (strongly agree). Strongly agree and agree answers for each question were considered as having a positive attitude for the respondent towards pharmacovigilance teaching.

After gaining the school ethical approval (1/11/16) to carry out the study, an invitation letter was sent to all Jordanian universities that provide a pharmacy program. The questionnaire was then distributed manually to the academics in Jordanian schools of pharmacy. Schools which accepted to participate in the study were visited once during the study period. All members who had either Masters or PhD degrees and who were available at the time of the school visit were invited to take part in the survey. Respondents were assured of the anonymity of the study and participation to the study was voluntary, i.e., completion and return of the questionnaires by the participants implied their consent. Our inclusion criteria were (1) Pharmacy academics in a pharmacy school in Jordan, (2) a PhD or Master degree holder, and (3) a staff member. Our exclusion criteria were (1) Lab supervisors even if they were master degree holders and (2) academics in the pharmacy schools whose major was not pharmacy.

Data Analysis

The data was analyzed using SPSS software version 19.0. Descriptive statistics were used to determine the mean and standard deviation (SD) for continuous variables and percentage for qualitative variables. The relationship between demographics (age, gender, education level, site of work, current position, specialty, and pharmacy practicing) and academics' familiarity with pharmacovigilance was assessed using the chi-square test. A P value of less than 0.05 was considered statistically significant.

Results

Demographics

Invitation letters were sent to all universities that offer undergraduate pharmacy programs in Jordan (total = 14). Eight universities out of the approached universities accepted to take part in the present study with total responses of 87 pharmacy school members. The total number of academics in the participated universities was 280 members and each pharmacy school member available at the time of the school visit was asked to fill out the questionnaire delivered by hand with a response rate of 31.1%.

The demographic characteristics of the respondents are shown in Table 1. The majority of participants were aged between 35 and 44 years, and the average years of teaching were 8.1 years. In this study, females accounted for 63.1% (n = 53) of participants.

Table 1: Demographic characteristics of the study sample (n=87)

Parameter		Result (Mean ± SD) (Number (%))
Age; N (%)		
	25-34 years	26 (29.9%)
	35-44 years	43 (49.4%)
	45-54 years	12 (13.8%)
	≥55 years	6 (6.9%)
Gender; N (%) Missing=3		
	Male	31 (36.9%)
	Female	53 (63.1%)
Educational level; N (%)		
	PhD	68 (78.2%)
	Masters	19 (21.8%)
Site of work; N (%)		
	Public university	39 (44.8%)
	Private university	48 (55.2%)
Total years of teaching experience		8.09±7.46
Current position; N (%)		
	Professor	9 (10.3%)
	Associate professor	9 (10.3%)
	Assistant professor	50 (57.5%)
	Instructor, teaching assistant	19 (21.8%)
Specialty; N (%)		

Parameter		Result (Mean ± SD) (Number (%))
	Pharmaceutical science	33 (37.9%)
	Medicinal chemistry	17 (19.5%)
	Pharmacognosy	5 (5.7%)
	Clinical pharmacy	31 (35.6%)
Pharmacy practicing; N (%)		
	Yes	55 (63.2%)
	No	32 (36.8%)

Pharmacy School Academics' Familiarity Regarding Pharmacovigilance and ADR Reporting

Of the responding pharmacists, 87.4% heard about pharmacovigilance and 79.3% were aware about the exact definition of pharmacovigilance. Only 26.4% of the participating pharmacists attended a workshop about pharmacovigilance. About half of the participants did not know about the presence of pharmacovigilance centers in Jordan and an official standardized form for reporting adverse drug reactions (43.7% and 49.4%, respectively). The results are presented in Table 2.

Table 2: Teaching fellows' knowledge toward pharmacovigilance concept and policy

Question	No. of respondents (%)
Have you heard about the concept of pharmacovigilance? (yes)	76 (87.4%)
Do you know the definition of pharmacovigilance? (yes)	69 (79.3%)
Have you ever had a course or attended a workshop about pharmacovigilance? (yes)	23 (26.4%)
Do you know that there is a pharmacovigilance center in Jordan? (yes)	38 (43.7%)
Do you know that whether there is an official standardized form for reporting adverse drug reactions in Jordan? (yes)	43 (49.4%)
Do you have sufficient knowledge to educate the students how to report adverse drug reactions to the relevant authorities in Jordan? (yes)	32 (36.8%)

No significant correlation was found between familiarity with the pharmacovigilance concept and policy among pharmacy academics in the Jordanian universities and age, gender, educational level, or current position. However, knowing about the official standardized form for reporting adverse drug reactions in Jordan had statistically significant differences between different specialties (pharmaceutical science, medicinal chemistry, pharmacognosy, and clinical pharmacy; $p = 0.005$). Clinical pharmacists showed the best awareness between specialties as shown in Table 3. The answers of the remaining questions on pharmacovigilance familiarity were not statistically significantly different between academics' specialties.

Academics who work at public universities showed higher frequencies of familiarity regarding the presence of pharmacovigilance centers in Jordan and the ADR reporting form compared with pharmacists at private universities (p value = 0.01 and 0.04, respectively). The results are shown in Table 3.

Table 3: Comparison of pharmacovigilance knowledge between different specialties of teachers and different sites of work

Questions*	Pharmaceutical science (N=33) N (%)	Medicinal chemistry (N=17) N (%)	Pharmacognosy (N=5) N (%)	Clinical pharmacy (N=31) N (%)	P value†	Public universities (N=39) N (%)	Private universities (N=48) N (%)	P-value†
Have you heard about the concept of pharmacovigilance? Yes	27(82%)	15(88%)	5(100%)	28(90%)	0.60	37(95%)	39(81%)	0.06
Do you know the definition of pharmacovigilance? Yes	24(73%)	12(71%)	5(100%)	27(87%)	0.30	31(79%)	38(81%)	0.87
Have you ever had a course or attended a workshop about pharmacovigilance? Yes	8(24%)	4(24%)	1(20%)	9(29%)	0.95	13(33%)	10(21%)	0.19
Do you know that there is a pharmacovigilance center in Jordan? Yes	12(36%)	5(29%)	2(40%)	19(61%)	0.12	23(59%)	15(31%)	0.01‡
Do you know that there is an official standardized form for reporting adverse drug reactions in Jordan? Yes	15(45%)	3(18%)	3(60%)	22(71%)	0.005‡	24(62%)	19(40%)	0.04‡
Do you have sufficient knowledge to educate the students how to report adverse drug reactions to the relevant authorities in Jordan? Yes	10(30%)	3(18%)	3(60%)	15(48%)	0.10	18(46%)	14(29%)	0.10

*Question answers were either Yes or No.

†Significant when P value < 0.05

‡Significantly different

Table 4 shows that pharmacists who had experience in the practice of pharmacy at community or hospital pharmacies have higher degree of sufficient information to educate the students on how to report adverse drug reactions than pharmacists who did not have experience in pharmacy practice (p value = 0.008).

Table 4: Pharmacovigilance knowledge between practiced and not practiced pharmacists

Questions*	Practiced (N=55) N (%)	Not practiced (N=32) N (%)	P value†
Have you heard about the concept of pharmacovigilance? Yes	50(91%)	26(81%)	0.19
Do you know the definition of pharmacovigilance? Yes	46(84%)	23(72%)	0.13
Have you ever had a course or attended a workshop about pharmacovigilance? Yes	16(29%)	7(22%)	0.46
Do you know that there is a pharmacovigilance center in Jordan? Yes	20(36%)	18(56%)	0.08

Questions*	Practiced (N=55) N (%)	Not practiced (N=32) N (%)	P value†
Do you know that there is an official standardized form for reporting adverse drug reactions in Jordan? Yes	29(53%)	14(44%)	0.41
Do you have sufficient knowledge to educate the students how to report adverse drug reactions to the relevant authorities in Jordan? Yes	26(47%)	6(19%)	0.008‡

*Question answers were either Yes or No.

†Significant when P value < 0.05

‡Significantly different

Pharmacy School Academics' Attitude toward Pharmacovigilance Teaching

Table 5 includes attitudes of pharmacy school academics in Jordan toward pharmacovigilance teaching. Only two academics disagreed with the necessity to include pharmacovigilance as a core topic in pharmacy education and teaching pharmacy students how to report ADRs (2.4%). The majority of the participating academics (87.2%) either agreed or strongly agreed that the pharmacist has the responsibility toward ADR reporting. Fifty-seven academics (66.3%) claimed that pharmacy students can perform ADR reporting during their clerkship. A significant difference (P = 0.031) was found in responses according to current academic positions of the academics. Only 17.7% (n = 15) of the academics either agreed or strongly agreed that the topic of pharmacovigilance is well covered at pharmacy schools currently.

Table 5: Attitudes of pharmacy school teaching fellows in Jordan toward pharmacovigilance teaching (n=87)

Questions	Strongly disagree N (%)	Disagree N (%)	Neither agree nor disagree N (%)	Agree N (%)	Strongly agree N (%)	Age	Gender	Position	Specialty	University type	Practiced
						P- value†					
Pharmacovigilance should be included as a core topic in pharmacy education, missing=2	0(0%)	2 (2.4%)	4 (4.7%)	32 (37.6%)	47 (55.3%)	0.436	0.432	0.676	0.663	0.452	0.687
The topic of pharmacovigilance is well covered in current pharmacy school curricula, missing=2	6 (7.1%)	25 (29.4%)	39 (45.9%)	14 (16.5%)	1 (1.2%)	0.997	0.455	0.832	0.759	0.607	0.222
Pharmacists more than other health care professionals hold the responsibility toward adverse drug reactions reporting, missing=1	0 (0%)	3 (3.5%)	8 (9.3%)	39 (45.3%)	36 (41.9%)	0.392	0.128	0.837	0.251	0.704	0.595
Information on how to report adverse drug reactions should be taught to pharmacy students prior to graduation, missing=1	0 (0%)	2 (2.3%)	1 (1.2%)	42 (48.8%)	41 (47.7%)	0.052	0.569	0.269	0.793	0.459	0.502
Pharmacy students can perform adverse drug reactions reporting during their clerkship, missing=1	0 (0%)	6 (7%)	23 (26.7%)	38 (44.2%)	19 (22.1%)	0.061	0.683	0.031*	0.437	0.758	0.404

†Significant when P value < 0.05 *Significant difference

Discussion

This is the first study that assessed the level of awareness about pharmacovigilance among academics in Jordanian schools of pharmacy and explored the level of pharmacovigilance education provided to pharmacy students in their undergraduate study, from the point of view of academics. Previous studies in the Middle East including Jordan targeted either healthcare professionals (i.e., doctors, nurses, and pharmacists) or students but not academics [15-19].

The results of the study showed that the majority of pharmacy academics has good familiarity and awareness about the pharmacovigilance concept and policy. They heard about the pharmacovigilance concept (87.4%) and were aware about the exact definition of pharmacovigilance (79.3%). This finding is similar to the results of previous studies conducted among healthcare professionals (Abdel-Latif and Abdel-Wahab, 2014; Almandil, 2016) where they found that pharmacists rather than other healthcare professionals had a good knowledge and awareness of pharmacovigilance and ADR concept [20, 21].

Suyagh, *et al.* (2015) studied the pharmacovigilance awareness among Jordanian pharmacists in the community and hospitals [9]. However, their results were in contrast to our results. They found that the majority of community and hospital pharmacists have insufficient awareness and poor knowledge about pharmacovigilance. This variation in knowledge may be explained by the differences in the respondents' type; that is, in the current study, the respondents were academics in the pharmacy schools who are always in direct contact with the recent knowledge. Moreover, the former study was carried out four years ago, and since then the familiarity of the concept of pharmacovigilance has spread among healthcare professionals.

Abu Hammour et al. (2017) also found that most healthcare professionals were not aware of the concept of pharmacovigilance [22] while Mukattash et al. (2018) specified the lack of knowledge about pharmacovigilance and ADR reporting among pediatricians [23].

This study found that a quarter of the participating academics had attended a workshop about pharmacovigilance (26.4%) whereas the results from Suyagh *et al* (2015) were only 8.2% of practicing pharmacists in Jordan had attended pharmacovigilance workshops [9]. This may be due to the interest of academics more than others toward workshops and training programs in different pharmacy topics.

More than half of the participants did not know about the presence of pharmacovigilance centers in Jordan and an official standardized form for reporting ADRs. This is similar to the results of Suyagh *et al.* (2015) and Abdel-Latif and Abdel-Wahab (2014) which showed that most pharmacists were not aware of the availability of legal provisions that provide pharmacovigilance activities, i.e., 63.1% and 75%, respectively [9,20].

To the best of our knowledge, this is the first study to compare the degree of pharmacovigilance familiarity among pharmacy academics in Jordanian universities with other variables such as age, gender, educational level, site of work, current position, specialty, and pharmacy practicing. The present study found no significant correlation between familiarity with pharmacovigilance concept and policy and age, gender, educational level, and current position. Nevertheless, clinical pharmacists showed the best awareness of the different specialties regarding knowledge of the presence of pharmacovigilance centers in Jordan ($p = 0.005$). This is because of the nature of the practice of clinical pharmacists in health care settings where they have frequent and regular interactions with health care professionals, contributing to better and safer medical care for patients [24].

Pharmacists who work at public universities showed higher frequencies of awareness about the presence of pharmacovigilance centers in Jordan and the ADR reporting form compared with pharmacists at private

universities (p value = 0.01 and 0.04, respectively). One of the reasons is that most public universities in Jordan have their own teaching hospital. Thus, pharmacists are always exposed to the practice of reporting ADRs. Another reason could be that one of the pharmacovigilance centers in Jordan is located in a pharmacy school at one of the public universities.

Interestingly, pharmacists that had experience in the practice of pharmacy at a community or hospital pharmacy have higher degree of sufficient information to educate the students on how to report ADRs than pharmacists who did not have experience in the practice of pharmacy (p value = 0.008). This finding was expected since practiced pharmacists might have the chance to experience ADR reporting during their work.

This study shows that pharmacy schools provide inadequate coverage about pharmacovigilance from the point of view of academics and ADR reporting in their undergraduate curricula, suggesting that a comprehensive curriculum related to pharmacovigilance should be considered and implemented.

Almost all of the academics (92.9%) agreed (and strongly agreed) that pharmacovigilance should be included as a core topic in pharmacy education, and 96.5% agreed (and strongly agreed) that there is a need to teach pharmacy students how to report ADRs prior to graduation. This finding was also noticed in the Abu Hammour study (2018) that found that despite the low level of awareness about pharmacovigilance among healthcare professionals, the majority believed in the necessity of reporting ADRs [22]. Meeting this need will require schools to provide guidance and training programs on ADR reporting protocol.

There was a significant difference in the mean scores of the ability of pharmacy students to perform ADR reporting during their clerkship between different academic positions where instructors who supervise clinical clerkships and training rotations and teaching assistants believed that the current students are not capable of reporting ADRs. Their opinion is based on their role as preceptors for students; they are usually present with students during training in hospitals.

The vast majority of academics (87.2%) agreed (and strongly agreed) that the pharmacist is the most important healthcare professional to report ADRs. These findings are consistent with the results for healthcare professionals in other studies [24-27].

The sample size of the study was relatively small which limits the generalizability of the findings. Another limitation was the close ended and insufficient number of questions that assessed the actual knowledge about pharmacovigilance among participants. However, we can judge that the present study can be the base for a comprehensive large-scale study to evaluate the actual knowledge and attitudes toward pharmacovigilance among academics in Jordan.

Conclusion

Although the study showed that pharmacy school academics in Jordan have good familiarity with the pharmacovigilance concept and definition, there was insufficient knowledge about the availability of pharmacovigilance centers and reporting protocol. However, with the agreement of the majority of participants, we recommend adding topics in pharmacovigilance and the methods of detecting, preventing, and reporting ADRs to the current pharmacy school curricula.

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مدى إلمام والموقف الأكاديمي للصيدلة في الجامعات الأردنية تجاه اليقظة الدوائية: دراسة مقطعية

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ملخص

المقدمة: يزيد الوعي باليقظة الدوائية بين أكاديميي الصيدلة من معرفة الطلاب وخريجي الصيدلة ومهاراتهم في الإبلاغ عن التفاعلات الدوائية الضارة.

الغرض: تهدف هذه الدراسة إلى تقييم مستوى الوعي حول اليقظة الدوائية بين الأكاديميين في كليات الصيدلة في الأردن وتقييم التعليم الحالي لموضوع اليقظة الدوائية من وجهة نظر الأكاديميين.

الطريقة: تم توزيع استبيان ورقي مكون من 24 فقرة على ثماني كليات للصيدلة أبدت موافقتها بالمشاركة.

النتائج: تم إكمال الاستبيان من قبل 87 أكاديميًا للصيدلة. كان معظم المشاركين على دراية بمفهوم اليقظة الدوائية (79.3٪) ووافقت الغالبية (96.5٪) على تطبيق تقارير التأثيرات السلبية للأدوية في المناهج المدرسية. على الرغم من هذا الموقف الإيجابي، كان 26.4٪ فقط من المشاركين قد حضروا سابقًا ورشة عمل حول اليقظة الدوائية و36.8٪ تمكنوا من تثقيف الطلاب حول كيفية الإبلاغ عن التفاعلات الدوائية.

الخلاصة: الأكاديميون في كلية الصيدلة في الأردن لديهم معرفة جيدة وسلوك إيجابي حول اليقظة الدوائية. ومع ذلك، يوصى بإدراج موضوعات حول الكشف عن التفاعلات الدوائية الضارة ومنعها والإبلاغ عنها في المناهج الدراسية الحالية.

الكلمات الدالة: التفاعلات الدوائية الضارة، اليقظة الدوائية، التعليم الصيدلاني.

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Agranulocytosis: A rare side effect of carbimazole and the function of Cholestyramine in Hyperthyroidism

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ABSTRACT

Agranulocytosis is a rare side effect of antithyroid drugs that usually develops within the few months after starting treatment. We report a 45-year-old Indian female who presented to the hospital with shortness of breath, lethargy, decreased appetite, pharyngitis, and fever after used of Carbimazole 30mg OD for 2 months due to hyperthyroidism which was prescribed by her clinician. Her full blood count revealed neutropenia with a count of 0.03 × 109/L. Carbimazole was discontinued and she was given antibiotics. Cholestyramine was used to treat her hyperthyroidism. In conclusion, agranulocytosis induced by the Carbimazole is important to recognise and treat early to prevent morbidity and mortality.

Keywords: Carbimazole, Hyperthyroidism, Cholestyramine, Agranulocytosis, Neutropenia

INTRODUCTION

Hyperthyroidism is a very common disease, most likely caused secondary to Graves's disease followed by toxic multi-nodular goitre. Thioamide drugs are one of the medicine categories used in the treatment of hyperthyroidism. It inhibits the thyroid peroxidases that catalyze the iodination of tyrosine residues in thyroglobulin and the oxidative coupling of iodinated tyrosines. Inhibition of iodination is competitively antagonized by iodide at low drug concentrations, but not at higher drug concentrations [1]. It has been suggested that it also reduces the autoimmunity that underlies the Graves' disease. Thioamides, which have been in use for more than half a century, remain cornerstones in the management of hyperthyroidism [2]. Most patients tolerate treatment well, but some may develop life-threatening side effects such as agranulocytosis [3,4,5]. Agranulocytosis is the most severe adverse hematologic reaction associated with the Thioamides. Agranulocytosis typically develops within the first 3 months of treatment, although it can occur at any time and as late as 12 months after starting Thioamide therapy [6].

The prevalence of agranulocytosis is about 0.2-0.5%. The risk factors for agranulocytosis are unknown [7]. There is no predilection for either gender, and the reaction may be idiosyncratic, or dose related. Some reports suggest that patients older than 40 years or those taking high dosages of methimazole (e.g., >40 mg/day) might be more susceptible than those on any dosage of propylthiouracil [8]. If agranulocytosis is diagnosed, the drug should be discontinued, the patient monitored for signs of infection, and antibiotics instituted if necessary [9]. Although some cases of granulocytopenia have resolved with substitution or continuation of Thioamides, the risks of drug re-challenge clearly outweigh the benefits, and other treatments should be instituted [2].

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Case Report:

A 45-year-old Indian woman with the known past medical history of hyperthyroidism was hospitalized in

Malaysia hospital with shortness of breath, lethargy, decreased appetite, pharyngitis, and fever. She suffered also from major weight loss; her weight dropped down from 56 Kg to 47 Kg within 2 months. This incidence encouraged her to see a doctor and got the diagnosis of hyperthyroidism in April 2019. She denied any history of palpitation, sweating, or diarrhoea. She denied any allergy to medications. Her vital signs showed BP 92/58 mmHg, heart rate 132 bpm, respiratory rate 20 breaths/min, oxygen saturation 97%, weight 47 kg, and height of 161 cm. The patient is a non-smoker, non-alcoholic and does not abuse drugs. She is married and has one son (12 years old). Her occupation was a fruit seller. Her past medication history consists of once-daily Propranolol 20 mg and Carbimazole 30 mg.

Her blood test showed the values for Hemoglobin 8.2 mg/dl, total leukocyte counts 1240/mm ^{3}, platelet 166,000 per µL, SCr 47 µmol/L, K 3 mEq/L, PO4 0.3 mmol/L, and Albumin 20 g/litre. Differential leukocyte counts - Neutrophil 3 %, Lymphocytes 80 %, Monocytes (M) 2 %, Basophils 0 %, peripheral blood smear showed normocytic normochromic RBC series, reduced total leukocyte count with neutropenia. Initial thyroid function test showed TSH value of 0.27 mlU/L (0.4 - 4.5 mlU/L), FT4 of 30.47 pmol/L (9.0-24.0 pmol/L), and FT3 of 1.93 pmol/L (2.2 - 5.4 pmol/L).

The patient was diagnosed with neutropenic sepsis secondary to carbimazole induced agranulocytosis. As Carbimazole was the drug responsible for current patient status, it was discontinued, and she was treated in the ward from 9 to 24/06/2019 as shown in Table 1.

Table 1.Medication history of the patients from 9 to 24/06/2019

Date	Name of the drug (Brand/Generic)	Dose	Route	Frequency	Stop date
09/06/2019	Rocephin (ceftriaxone)	2g	IV	OD	10/06/2019
10/06/2019	Tazocin (Piperacillin/tazobactam)	4.5g	IV	QID	24/06/2019
20/06/2019	Propranolol	20mg	Oral	OD	
22/06/2019	Cholestyramine	4g	Oral	TDS	
13/06/2019	Vit.C		Oral	OD	
18/06/2019	Lithium	350 mg	Oral	BID	21/06/2019
12/06/2019	Ferrous Fumarate	200 mg	Oral	BID	
10/06/2019	Dexamethasone	2 mg	Oral	QID	10/06/2019
09/06/2019	Lugols Solution		Oral	TDS	10/06/2019
10/06/2019	KH2PO4		IV	OD	12/06/2019
09/06/2019	MgSo4		IV	BID	11/06/2019
09/06/2019	Propylthiouracil (PTU)	600 mg	Oral	TDS	09/06/2019
09/06/2019	Hydrocortisone	200 mg	Oral	TDS	11/06/2019
11/06/2019	Noradrenaline		IV	OD	11/06/2019
09/06/2019	Paracetamol	1 g	Oral	OD	24/06/2019
09/06/2019	Thiamine	200 mg	IV	OD	10/06/2019
11/06/2019	KCL	1 mg in 100 cc	IV	OD	11/06/2019
12/06/2019	0.2% chlorohexidine		Oral	TDS	24/06/2019
12/06/2019	Slow K	1.2 g	IV	BID	12/06/2019
09/06/2019	Rocephin (ceftriaxone)	2g	IV	OD	10/06/2019
09/06/2019	MgSo4		IV	BID	11/06/2019
09/06/2019	PTU	600 mg	Oral	TDS	09/06/2019

Date	Name of the drug (Brand/Generic)	Dose	Route	Frequency	Stop date
09/06/2019	Hydrocortisone	200 mg	Oral	TDS	11/06/2019
09/06/2019	Lugols Solution		Oral	TDS	10/06/2019
09/06/2019	Paracetamol	1 g	Oral	OD	24/06/2019
09/06/2019	Thiamine	200 mg	IV	OD	10/06/2019
10/06/2019	Tazocin (Piperacillin/tazobactam)	4.5g	IV	QID	24/06/2019
10/06/2019	Dexamethasone	2 mg	Oral	QID	10/06/2019
10/06/2019	KH2PO4		IV	OD	12/06/2019
11/06/2019	Noradrenaline		IV	OD	11/06/2019
11/06/2019	KCL	1 mg in 100 cc	IV	OD	11/06/2019
12/06/2019	Ferrous Fumarate	200 mg	Oral	BID	
12/06/2019	0.2% chlorohexidine		Oral	TDS	24/06/2019
12/06/2019	Slow K	1.2 g	IV	BID	12/06/2019
13/06/2019	Vit.C		Oral	OD	
20/06/2019	Propranolol	20mg	Oral	OD	
22/06/2019	Cholestyramine	4g	Oral	TDS	

Her condition improved within seven days of stopping the Carbimazole. On 17/06/2019 total white blood cell and neutrophil counts reverted to near normal range and symptoms like shortness of breath, lethargy, decreased appetite, pharyngitis, and fever disappeared. The patient was discharged from the hospital after 14 days and her discharge medications are shown in Table 2.

Table 2. Patient discharged medications list

S. No	Drug	Dose	Frequency
1.	Cholestyramine	4 g	TDS
2.	Prednisone (0.5/kg)	20 mg	OD
3.	Propranolol	20 mg	OD
4.	Ferrous Fumarate	400 mg	OD
5.	Vit.C	100 mg	OD
6.	Vit.B	10 mg	OD
7.	Folate	5 mg	OD

Discussion:

Agranulocytosis is a rare but serious complication of antithyroid drug therapy. A study done by Van der Klauw *et al* [9]reported a relative risk of agranulocytosis among 115 for patients who received the antithyroid drugs (ATD), was found the highest risk among all others evaluated pharmacological agents. Similarly, in a study done by Tajiri *et al*., among 15,398 Japanese patients with Graves' disease, there was no difference in the incidence of agranulocytosis between patients receiving propylthiouracil and those receiving the methimazole [10]. The result of this case report is consistent with Van der Klauw et al and Tajiri et al [9,10].

, A study done by Nakamura *et al* [11] reported an analysis of 754 cases that published of ATD induced agranulocytosis in Japan[11], the mean age of onset was 43.4

± 15.2 years and indicated that the females were more affected than males (6.3:1 ratio). Another, study done by Yang *et al* [12] reported an analysis of 114 cases with ATD induced agranulocytosis diagnosed in a single Chinese centre revealed a higher female-to-male ratio (10.4:1) and similar age of onset (41.7 ± 12.3 years) [12]. Agranulocytosis usually develops in the first 3 months after antithyroid drugs therapy is initiated [11]. In Japan, a 754 retrospectively reviewed cases of agranulocytosis after use of ATD found that more than 70% of patients who developed this side effect within 2 months, and nearly 85% showed this effect within 3 months[9]. The current case report of agranulocytosis manifest with 45 year old female patient and this consistent with the finding of Nakamura *et al*[11] and Yang *et al*[12].

The current case may have a difference in time of onset and may be related to the disease mechanism, with the immune-mediated process that leads to the more rapid destruction of neutrophils as opposed to direct toxicity. The previous studies recognised that the mean duration of treatment with propylthiouracil, carbimazole and methimazole needed to cause agranulocytosis was found to be 36, 41, and 42 days, respectively [13]. Agranulocytosis can manifest not only after the first treatment with ATD but also in later courses. It can manifest up to eight courses later (with either the same or a different ATD) but usually occurs 5 months after finishing the previous treatment [14]. Another study done by Kim *et al* reported severe agranulocytosis developed after 3 weeks on carbimazole treatment[15]. In the present case report, agranulocytosis manifests 2 months after treatment with Carbimazole 30 mg OD. In summary, Agranulocytosis is life-threatening, but treatable within the opportunity, and the most important lesson for physicians in the future is to remain vigilant for ATD induced agranulocytosis regardless of treatment duration or dose.

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ندرة المحببات: تأثير جانبي نادر للكربيمازول ووظيفة الكوليستيرامين في فرط نشاط الغدة الدرقية

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ملخص

ندرة المحببات هو أحد الآثار الجانبية النادرة للأدوية المضادة للغدة الدرقية التي عادة ما تتطور في غضون بضعة أشهر بعد بدء العلاج. أبلغنا عن امرأة هندية تبلغ من العمر 45 عامًا قدمت للمستشفى مع ضيق في التنفس والخمول وتراجع الشهية والتهاب البلعوم والحمى بعد تناولها لـ كاربيمازول 30 ملغ مرة يوميا لمدة شهرين بسبب فرط نشاط الغدة الدرقية الذي وصفه الطبيب السريري لها. وعند كشف تعداد دمها الكامل وجد قلة العدلات بنسبة 0.03 × 109 / لتر. تم إيقاف كاربيمازول وتم إعطاؤها المضادات الحيوية. تم استخدام كوليستيرامين لعلاج فرط نشاط الغدة الدرقية لها بدلاً من كاربيمازول. في الختام ، ندرة المحببات التي يسببها كاربيمازول من المهم التعرف عليها والعلاج في وقت مبكر لمنع المراضة والوفيات.

الكلمات الدالة: كاربيمازول ، فرط نشاط الغدة الدرقية ، كوليستيرامين ، ندرة المحببات ، قلة العدلات.

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Essential Oil of *Salvia officinalis* L. from the Algerian Saharan Atlas: Chemical Composition and Biological Evaluation

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ABSTRACT

In this study, chemical composition, and biological activities of the essential oil of *Salvia officinalis*, a native plant cultivated in Laghouat (Algerian Sahara), were studied. Chemical composition of the essential oil was identified by gas chromatography/mass spectrometry (GC/MS). Thirty-nine components representing 96.41% of the essential oil were detected with camphor (16.41%), α-thujone (15.68%), manool (15%), viridiflorol (11.69%) and 1,8-cineole (10.06%) as the major compounds. Antioxidant activity was employed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging. The essential oil's IC_{50} value was 0.222 mg /mL in the DPPH assay which could be regarded as reasonable antioxidant power. The antimicrobial activities were evaluated on selected Gam-positive and Gram-negative bacteria, as well as on two pathogenic fungi. The results revealed agreeable and broad-spectrum antibacterial activity while the oil demonstrated weak antifungal potential. On the other hand, the antiproliferative potential of the oil was assessed on different human cancer cell types with the oil's activities on leukemia and prostate cancers being reported for the first time in literature. The LD_{50} values of the oil were in the 200-400 µg/mL on the different cancer types examined. These findings may encourage further investigations in the potential use of *S. officinalis* oil as naturally occurring bioactive ingredient for food and pharmaceutical industry.

Keywords: *Salvia officinalis*, essential oil, chemical composition, antioxidant, antiproliferative, antimicrobial.

1. INTRODUCTION

The family of Lamiaceae consists of about 230 genera and 7100 species worldwide. Many species from the Lamiaceae family are considered of high importance because of their uses in medicine (**1**). The genus Salvia includes approximately 900 species that are widely growing throughout the Mediterranean (**2**). In Algeria, 23 Salvia species are growing, among which *Salvia officinalis* (common sage) is the most common species of the genus (**3,4**).

Salvia officinalis is a perennial round shrub; its leaves and flowering tops have strong aromas and are used to produce essential oils. Compared to other species of Salvia, *S. officinalis* is considered to have the highest abundance of volatile oils (**5**). Since antiquity, this plant has been recognized for its medicinal significance (**6**). It is used in folk medicines for antibacterial, antitumor, antioxidant and anti-inflammatory treatments, as well as for a range of diseases including those of the nervous system, heart and blood circulation, the respiratory, digestive, metabolic, and endocrine system (**7, 8, 9,10,11**).

Phytochemical studies of *Salvia officinalis* revealed a great number of bioactive compounds possessing a variety of biological activities. Interestingly, **S. officinalis** is considered to have the highest amount of essential oil compared to the other species of **Salvia** (**5**). *Salvia*

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officinalis essential oil has various compositions depending on the genetic, climatic, season, and environmental factors (**12**). The purpose of the current study was to identify the chemical composition of the essential oil extracted from *Salvia officinalis* grown in Algeria from the Laghouat region via GC-MS technique and to determine the antioxidant activity along with other biological activities associated with the essential oil.

Materials and Methods

Plant Material

The aerial parts of *S. officinalis* were collected during the flowering stage in July 2016 from the Laghouat region of Algeria (latitude 33°47'59''N longitude 2°51'54''E and altitude 764 m). The plant material was taxonomically identified by the botanical survey, and the voucher specimens (LGP So/07/16) were deposited in the laboratory of Process Engineering, University of Laghouat (Algeria).

Extraction of Essential Oil

The aerial parts (100 g) of *S. officinalis* were subjected to hydrodistillation for 3 h with 500 mL of distilled water using a Clevenger-type apparatus. The prepared volatile oils were dehydrated over anhydrous sodium sulphate and stored in dark vials in refrigerator at 4°C until analyzed. The yield was expressed in percentage.

Essential Oil Gas Chromatography-Mass Spectrometry Analysis

The essential oil analysis was performed on a chromatography's type Hewlett-Packard HP 7890 equipped with a capillary column HP-5MS (30 m × 0.32 mm, i.d., 0.25 µm film thicknesses) coupled to a mass spectrometer (MS) type with a Hewlett Packard 5975 detector impact of electrons, 70 EV. Oven temperature was held at 60°C for 8 min and increased from 60°C to 250°C at a rate of 2°C/min and held at 250°C for 20 min. Injector and detector temperatures were 250 and 280°C, respectively. Carrier gas was nitrogen at a flow rate of 1.2 mL/min in split mode 1:50 with an injection volume of 1 µL. The GC analysis was carried out using an Agilent 6890N GC system equipped with flame ionization detector (FID) operated at a temperature of 280°C. To obtain the same elution order of peaks detected by GC/MS, simultaneous injection on the GC was performed using the same column and appropriate chromatographic conditions as those described for the GC/MS system (**Table1**). Identification of the essential oil components was carried out by comparing their mass spectra and their KI (Kovats Index) with available databases (**13**). The percentage of each compound was computed using normalization method from the GC peak areas, calculated as mean values of three injections, without using correction factors.

Table 1: General information on GC-MS analysis performed.

Column type	HP-5MS (5% Phenyl, 95% dimethylpolysiloxane) 30m*0.32mm*0.25µm
Injection volume	1 µL
Injector tempereture	250 °C
detector temperature	280 °C
Mode of injection	Split 1:50
Vector gas	Helium

Determination of Antioxidant Activity

Radical scavenging activity of *S. officinalis* essential oil against DPPH radical was determined spectrophotometrically (**14**). The scavenging rate on DPPH radicals was calculated according to the formula:

$$Scavenging\ rate\ (\%)=[(A_0-A_1)/A_0]x100\%$$

where A_0 is the absorbance of the control solution, A_1 is the absorbance in the presence of samples in DPPH solution. The scavenging activity of the sample against DPPH radicals was expressed by IC_{50} value, defined as is the effective concentration at which DPPH radicals are scavenged by 50%, and is obtained by interpolation from

regression analysis (**14,15,16**).

Determination of Antimicrobial Activity

Microorganisms

The following microbial strains were obtained from the Microbial Culture Collection Centre of Medicine School at The University of Jordan: *Staphylococcus aureus* ATCC25923 (gram-positive bacterium), *Staphylococcus epidermidis* ATCC 12228 (Gram-positive bacterium), *Escherichia coli* ATCC 29425 (Gram-negative bacterium), *Pseudomonas aeruginosa* ATCC 15442 (Gram-negative bacterium), *Candida glabrata* ATCC 22553 (fungus), and *Candida albicans* ATCC10231 (fungus). The bacteria species were maintained in Mueller Hinton Agar and Triptic Soy Agar (MHA, TSA, Merck, Germany) whereas *Candida* spp. were maintained on Sabourand Dextrose Agar (SDA, Merck, Germany).

Minimum Inhibitory Concentration (MIC)

To assess the antimicrobial activities of the examined essential oil, the minimum inhibitory concentration (MIC) measurements, defined as the lowest concentration of the sample under investigation that inhibits bacterial or fungal growth after incubation at ideal temperature, were undertaken in 96 flat bottom microtiter plates (TPP, Switzerland) as formerly reported (**15**). Briefly, fresh overnight cultures of bacteria and yeasts were adjusted with media to an inoculum concentration of 1.0 x 10^5 CFU/per well. Positive controls, Ampicillin and Amphotericin B, and a negative control of untreated media were prepared under the same investigational conditions. Plates were incubated, with shaking, for 48h at incubation temperatures for the bacterial plates and the *Candida* plates of 37°C and 33°C, respectively. Optical densities were determined at wavelength 600 nm (OD_{600}) using a Microplate Reader (Palo Alto, CA, USA).

Determination of Antiproliferative Activity

Cells

All cell lines (MCF7, HeLa, PC3, and K562) were acquired from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM medium (Dulbecco's Modified Eagle's Medium), complemented with 10% Fetal Bovine Serum, 100 U/mL of Penicillin, 100 µg/mL of Streptomycin, at 37°C with 5% of CO_2. Viable cells count was resolved using the Trypan blue method as previously described (**16**).

MTT Assay

The antiproliferative activities of the examined essential oil was studied in 96-well round bottomed microplates employing the MTT assay (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl- tetrazolium bromide) (Sigma-Aldrich, USA) as previously described (**16**). In summary, a cell seeding density of $1x10^4$ cells/mL was used for all cells in 96 well plates. Cells were incubated for 24 h to allow attachment. The examined essential oil was applied at different concentration onto each well in triplicates and incubated for 48 h. Afterward, 10 µL of 0.5 mg/mL of MTT solution was added to each well and further incubated for 4 hours before measuring the absorbance at 570 nm. Growth inhibition was determined according to the following equation:

%inhibition= 100-(mean of Abs of test sample-mean of Abs of negative control)x100/(mean of Abs of positive control-mean of Abs of negative control)%

For data analysis, the Graph Pad Prism 8 software was used to calculate the inhibition percentage and results were presented as LD_{50} value, regarded as the concentration that concedes 50% growth suppression. Doxorubicin was employed as a positive control under the same experimental conditions as for the test samples.

Data Analysis

All measurements were performed in triplicates, with the results expressed as mean ± SD of three independent experiments (n=9). The means were statistically compared using one-way ANOVA, applying a Student's t-test, with $\alpha = 0.05$. The analyses were carried out using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, New York, NY, USA).

Results and Discussion

Yield and Composition of Essential Oil

The essential oil yield of *Salvia officinalis* aerial parts was 0.84 % (v/w based on dry weight) and the density of the concentrated oil was 0.93 g/mL. The oil had light yellow color, was soluble in methanol with the characteristic strong fragrance of sage. Several studies have reported different yields of *S. officinalis* oil from various regions; Constantine region (Algeria) 0.65% (**17**), Tunis 0.58% (**18**), Libya 0.4% (**19**), and Bulgaria 0.93 % (**20**). The reported differences in essential oil of *S. officinalis* yield from the different geographical regions may be attributed to diverse factors such as harvesting season, temperature and extraction techniques (**21**).

The GC-MS analyses resulted in the identification of 39 major and minor compounds, representing 96.41% of the total oil composition. The percentages, Kovats indices and the retention time of the identified compound of this essential oil were summarized in Table 2. The principal components of the essential oil are camphor (16.41%), α-thujone (15.68%), manool (15%), viridiflorol (11.69%) and 1,8-cineole (10.06%). β-caryophyllene (5.05%), β -thujone (4.20%), α–humulene (3.03%) and camphene (2.71%) were also present at significant concentrations. Other components were present at amount lower than 2 % of the total oil. Studies on *S. officinalis* performed in Morocco (**22**), Tunisia (**23**) and Libya (**19**) revealed significant variations in the chemical composition of *S. officinalis* essential oil relative to the findings of the current study. For example, the chemical composition of *S. officinalis* essential oil cultivated in Tunisia was reported to contain great amounts of camphor (25.14 %), while α-thujone, 1,8-cineole, and viridiflorol were found at (18.83 %,14.14 %,7.98 %) respectively (**23**). Interestingly, *S. officinalis* essential oil reported in this study has different composition from that reported by other Algerian studies. Noteworthy, Dob *et al.* (**24**) reported the chemical composition of *S. officinalis* essential oil from Algiers city, located in the north of Algeria, to contain camphor (20.4%), α-thujone (19.6%), 1,8-cineole (12.3%), β-thujone (8.0%), and viridiflorol (8.0%) as the major components of the oil. The reported variation in chemical composition of the oil is likely attributed to the different growth habitat, environmental factors, genetic variations, the growth phase of the plants and the extraction method (**12, 24, 25, 26**).

Table 2: Chemical composition of *Salvia officinalis* essential oil

N°	Retention time (mn)	Components	Kovats indices	Percentages (%)
1	3,4	Z-Salvene	855	0.06
2	5,2	Tricyclene	923	0.06
3	5,4	α-Thujone	931	0.21
4	5,6	α-Pinene	938	1.20
5	6,2	Camphene	953	2.71
6	7,3	Sabinene	973	0.26
7	7,4	2-β- Pinene	980	1.76
8	8,3	β-Myrcene	994	1.60
9	8,9	1-Phellandrene	1006	0.09
10	9,6	α -Terpinene	1012	0.23
11	10,2	p-Cymene	1027	0.36
12	**10,6**	**1,8-cineole**	**1030**	**10.06**
13	12,3	γ -Terpinene	1059	0.50
14	12,9	Z-Sabinenehydrate	1073	0.21
15	14,2	α -Terpinolene	1084	0.88
16	**15,7**	**α-Thujone**	**1105**	**15.68**
17	16,3	β - Thujone	1115	4.20
18	**18,2**	**Camphor**	**1144**	**16.41**
19	19,0	Isopinocamphone	1161	0.09
20	19,4	Borneol L	1165	0.55
21	19,5	Isoborneol	1156	0.36

N°	Retention time (mn)	Components	Kovats indices	Percentages (%)
22	20,2	4-Terpineol	1174	0.32
23	21,2	α –Terpineol	1198	0.17
24	27,7	borneol acetate	1273	1.43
25	28,3	Sabinyl acetate	1287	0.16
26	36,0	β-Caryophyllene	1409	5.05
27	38,1	α -Humulene	1444	3.03
28	38,4	Allo-aromadendrene	1478	0.08
29	39,6	α -Amorphene	1470	0.15
30	39,7	(-)-Germacrene D	1480	0.09
31	40,6	α -selinene	1493	0.18
32	41,7	γ -Cadinene	1513	0.06
33	42,4	β-Cadinene	1524	0.21
34	45,6	(-)-Caryophyllene oxide	1581	0.86
35	**46,4**	**Viridiflorol**	**1590**	**11.69**
36	46,8	6,12-Epoxyspiroax-4-ene	1588	0.19
37	49,0	α-Cadinol	1653	0.15
38	49,4	β-Eudesmol	1654	0.11
39	**69,8**	**Manool**	**2055**	**15.00**
		Total (%)		96.41

Antioxidant DPPH Activity

Antioxidant activity of the *salvia officinalis* essential oil has been determined by one test system; the DPPH assay. The IC_{50} values (the concentration reducing 50 % of DPPH) obtained for scavenging activity on DPPH radical are presented in Table 3. In the DPPH assay, the ability of the investigated essential oils to act as donors of hydrogen atoms or electrons in transformation of DPPH into its reduced form DPPH-H was investigated. In this assay essential oil has demonstrated a reasonable activity to scavenging and decolorate the radical DPPH with an IC_{50}= 0.222 mg/mL relative to the standard ascorbic acid IC_{50} =0.075mg/ml. Our results come in great consistency with previous literature reports that correlate the efficiency of the antioxidant power of an essential oil to its content of monoterpenes hydrocarbons and oxygenated monoterpenes (**25, 26**). Noteworthy, it appears that the antioxidant activity of sage oil maybe due to its content of a known strong antioxidant, as α-pinene and several other sesquiterpenes (**27, 28**) with possible contribution from minor and major components to exhibit this activity.

Table 3. Antioxidant activity of the essential oils from *S. officinalis* and positive control (ascorbic acid) using the DPPH assay.

Sample	IC_{50} (mg/ mL)
Essential oil	0.222±0.013
Ascorbic acid	0.075±0.010

Antimicrobial Activity

The obtained MIC values of the investigated oil are presented in Table 4. As demonstrated by the results, *S. officinalis* essential oil possessed comparable bactericidal activity against the examined gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and gram-negative bacteria (*Escherichia coli*, and *Pseudomonas Aeruginosa*), with MIC values ranging between 136-212 µg/mL, suggesting a broad antibacterial spectrum. In contrast, very weak antifungal activity has been observed on the candida species under investigation. The literature is rich with reports that show potent antibacterial activity associated the essential oils from different species of sage. Our findings are consistent with

the previous literature reports indicating the wide spectrum of sage oil's antibacterial activity (**5, 6, 8**). Interestingly, the activity was attributed to certain chemical components of the oil, such as 1,8-cineole, camphor, α – and β-thujone, borneol, and p-cymene, among others (**6**). It has been suggested that these chemical components may exert their antimicrobial effects through the disruption of bacteria or fungal membrane integrity (**6; 9**). In addition, the sage oil studied here appears to contain fair quantities of oxygenated monoterpenes which may contribute to its antimicrobial activity (**8**). The pathogens studied in this report represent some human pathogens that are known to infect man and animal and may result in food deterioration and contamination Therefore, the reported antimicrobial activities are likely to be attributed to synergistic effects between the variable major and minor constituents of the oil, proposing that sage oil may potentially be beneficial in food preservation.

Antiproliferative activity

Salvia species have been extensively investigated for their chemical composition and pharmacological profile. Recently, there has been a growing interest in the field of essential oils (EOs) for the search of new naturally occurring anticancer molecules. Generally, EOs exert more potent anticancer activities than their individual components due to synergism (**29, 30, 31)**. Until today the anticancer activities of the Algerian *S. officinalis* EO had not been reported in human prostate and leukemia cell lines. Therefore, this investigation aimed to investigate the effects of the essential oil on human breast, cervix, prostate, and leukemia cancer cells. Results of the antiproliferative activity are summarized in Table 4. As can be seen, *S. officinalis* EO exhibited antiproliferative activity against all examined cancer cells, including the leukemia and prostate cancer cells, after only 48 hours of treatment with LD_{50} values of 214-363 µg/mL. Remarkably, the observed antiproliferative effects of the EO was possibly linked to its ability to infiltrate through the cell membrane. Morphological changes and modifications of the cell membrane were revealed after 48 hours of treatment using an inverted microscope.

Table 4. Antimicrobial (MIC) and antiproliferative (LD_{50}) activities of *S. officinalis* essential oil (mean ± SD).

Sample	Antimicrobial activity [a]						Antiproliferative activity[b]			
	S. epidermidis	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. glabrata***	***C. albicans***	**MCF-7**	**HeLa**	**PC3**	**K562**
Oil	140±9	136±11	168±17	212±12	420±13	512±18	214±10	219±13	230±11	363±9
Control	2 [a]	2 [a]	16 [a]	128 [a]	2 [a]	2 [a]	1[b]	5[b]	25[b]	25[b]
Student´s t-test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

(a) Antimicrobial activity of *S. officinalis* oil measured by MIC (µg/mL, mean ± SD). Results represent the means of three independent readings ±SD (n=9). Positive controls were Ampicillin (antibacterial) and Amphotericin B (antifungal) (**b**) Antiproliferative activity evaluation of *S. officinalis oil* by MTT assay in the examined human cancer cell lines, exposure time 48 h. The presented LD_{50} values are expressed as µg/mL ± SD and correspond to the means of three independent readings (n=9). Doxorubicin was the positive control anticancer agent. A Student´s *t*-test was used to determine the significant difference between two different samples, with $\alpha = 0.05$.

Considering these findings and the results reported elsewhere in the literature, further investigations should be encouraged to investigate the mechanism of action of alternative naturally occurring anticancer molecules from the Algerian *S. officinalis* EO.

Conclusion

Taken together, this study provides a full characterization of the chemical and biological profiles of the Algerian *S. officinalis* essential oil. The chemical composition of the oil revealed thirty-nine components

representing 96.41% of the essential oil with camphor (16.41%), α-thujone (15.68%), manool (15%), viridiflorol (11.69%) and 1,8-cineole (10.06%) as the major compounds. The oil appeared to have reasonable antioxidant properties compared to ascorbic acid in the DPPH assay. Broad spectrum antimicrobial activities were observed against the examined Gram-positive and Gram-negative bacteria. The oil exhibited growth inhibition properties against breast, cervical, leukemia and prostate cancer cell lines that could suggest a potential use for the oil as a nutraceutical for cancer prevention.

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التركيب الكيميائي والتقييم البيولوجي للزيت العطري لنبات *Salvia officinalis* L من الصحراء الجزائرية

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ملخص

في هذه الدراسة، تمت دراسة التركيب الكيميائي والأنشطة البيولوجية للزيت العطري من نبات *Salvia officinalis* ، وهو نبات محلي يزرع في الأغواط (الصحراء الجزائرية). تم تحديد التركيب الكيميائي للزيت العطري بواسطة الفصل الكروماتوجرافي الغازي / قياس الطيف الكتلي (GC / MS). تم اكتشاف 39 مكونًا يمثلوا 96.41٪ من الزيت العطري باستخدام الكافور (16.41٪) ، ألفا ثوجون (15.68٪) ، مانول (15٪) ، (11.69) viridiflorol٪ و (cineole 10.06)٪–1.8 كمركبات رئيسية. تم استخدام النشاط المضاد للأكسدة عن طريق ازالة الجزيئات الحرة من خلال 2،2–ثنائي فينيل 1–بيكريل هيدرازيل (DPPH). كانت قيمة IC_{50} للزيت العطري 0.222 مجم / مل في اختبار DPPH والتي يمكن اعتبارها قوة معقولة من مضادات الأكسدة. تم تقييم الأنشطة المضادة للميكروبات على بكتيريا مختارة موجبة الجرام وسالبة الجرام، وكذلك على نوعين من الفطريات الممرضة. أظهرت النتائج نشاطًا مضادًا للبكتيريا مقبول وواسع النطاق بينما أظهر الزيت إمكانات ضعيفة كمضاد للفطريات. من ناحية أخرى، تم تقييم إمكانات الزيت كمانع للتكاثر على أنواع مختلفة من الخلايا السرطانية البشرية مع تسجيل نشاط للزيت على اللوكيميا وسرطان البروستات لأول مرة في الدراسات. كانت قيم LD_{50} للزيت ما بين 200–400 ميكروغرام / مل على أنواع السرطان المختلفة التي تم فحصها. هذه النتائج تشجع على إجراء مزيد من الأبحاث عن الاستخدام المحتمل لزيت الميرمية العطري كمكون بيولوجي طبيعي نشط في صناعة الأغذية والأدوية.

الكلمات المفتاحية: *Salvia officinalis* ، الزيوت العطرية ، التركيب الكيميائي ، مضادات الأكسدة ، مضادات التكاثر، مضادات الميكروبات.

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Simultaneous Determination of Indapamide, Amlodipine Besylate and Perindopril Arginine Combined in Tablet Dosage Form Using High Performance Liquid Chromatography

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ABSTRACT

We developed and validated a high performance liquid chromatographic method for the simultaneous determination of a single-pill triple therapy containing: Indapamide, Amlodipine Besylate, and Perindopril. The validation parameters are tested by following the international conference of harmonization (ICH) guidelines. The three components were successfully separated in just 14 minutes. The aforementioned components were well-separated on an Inertsil C8- column (250mm x 4.6mm, 5µm) using a mobile phase prepared by mixing of Triethylamine counter ion solution (pH 3.0) with Acetonitrile at a fixed ratio of 2:1. Analysis was performed at a wavelength of 205 nm, eluent flow rate and column oven temperature was 1.5 ml/min, and 40°C, respectively. Linearity was observed in the concentration ranges of (20-30) µg/mL for Indapamide, and (80-120) µg/mL for both Amlodipine Besylate and Perindopril Arginine. We found the recovery percentages to be 99.98%, 101.04%, and 100.58% for Perindopril Arginine, Amlodipine Besylate, and Indapamide, respectively. Further, we found the detection limits to be 0.38 µg/mL, 0.99 µg/mL and 3.65 µg/mL, and the obtained quantitation limits were 1.16 µg/mL 3.01 µg/mL, and 11.06 µg/mL for Indapamide, Amlodipine Besylate, and Perindopril Arginine, respectively.

Keywords: Simultaneous Determination; Indapamide; Amlodipine Besylate; Perindopril Arginine; HPLC; Validation.

1. INTRODUCTION

Hypertension, a prevalent disease among 35-40 % of the adult population, is a major health concern in this century[1]. Hypertension negatively impacts the cardiovascular risk and is implicated to be the culprit in a plethora of severe and life-threatening ramifications which includes heart failure, myocardial infarction, and kidney damage[2]. Current guidelines advocate adopting evidence-based lifestyle modifications as a mean to manage elevated blood pressure. This includes engaging in regular aerobic type of exercise[3], reducing sodium intake[4, 5], stress and alcohol consumption[6]. However, the majority of clinical cases do require antihypertensive medications to lower blood pressure readings to safe levels. Therefore, a huge number of therapeutics has been introduced to the clinical practice to serve such a purpose. An accumulating body of research is now leaning toward promoting the usage of two or more anti-hypertensive

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agents formulated in one dosage form to perform their putative pharmacologic effect via different mechanisms of action[7]. Currently, it is widely accepted within the clinical practice settings to use a combination of two or more anti-hypertensive agents that are formulated in a single tablet given the enhanced efficacy and improved adverse reaction profile compared to the classical single agent dosage form. Even when the dose is doubled[7,8]. Diuretics, beta-blockers[9], calcium channel blockers[10], angiotensin-converting enzyme inhibitors[11] and angiotensin receptor blockers[12] are considered to be the five primary anti-hypertensive classes that are recommended by the American Heart Association and the European Society of Hypertension as an exclusive treatment. The use of combinations of multiple agents in a single pill formulation offers many benefits, among which, better patient compliance, improved adherence to treatment because patients prefer single treatment schedule more than multiple treatment schedules[12,13], faster response to treatment, minimized risk of side effect[13], and reduced cost of production[12].Recently, a tremendous attention was paid to the usage of a triple combination of three active components namely: indapamide (IN), perindopril (PEP)(perindopril arginine), and amlodipine(AM) (amlodipine besylate) to control high blood pressure[14]. Triplixam tablet is an example of a commercial single tablet formulation with triple combination of IN, PEP, AM and acts as an anti-hypertensive medicine.

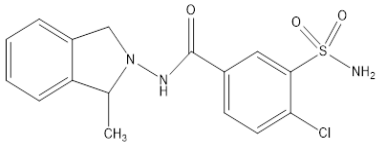
Indapamide (4-chloro-N-(2-methyl-1 -indoline-) 3-sulfamoylbenzamide) (figure 1a) is a known thiazide-like diuretic agent[15]. In addition to its classical effect of reducing intravascular space by promoting natriuresis and diuresis, Indapamide possesses an intrinsic ability to reduce vascular responsiveness to pressor amines (tyramine, tryptamine, and phenylethylamine)[16]. These Amines constrict the vascular system, and cause an increase in the heart rate and contractile force[15, 16].

Amlodipine (2-[(2-Aminoethoxy) methyl]-4-(2-chlorophenyl)-1, 4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid-3-ethyl-5-methyl ester)(figure 1b) is an agent known to antagonize calcium channels within the heart and the vessels. This feature is employed to control hypertension and to alleviate symptoms of ischemic heart disease such as chronic stable angina pectoris. Amlodipine is available in the market as a besylate salt[17].

Perindopril ((2S, 3aS, 7aS)-1-[(S)-N-[(S)-1-carboxybutyl]alnyl]hexahydro-2-indolinecarboxylic acid-1-ethyl ester)(figure 1c) is recognized as an angiotensin converting enzyme inhibitor and is widely used to treat hypertension and heart failure[18, 19,20, 21]. Perindopril is a prodrug that requires hydrolysis to its active metabolite, perindoprilat[19, 20]. Perindopril is available in the market as perindopril arginine or perindopril erbumine[19].

Several studies are directed toward determining of AM, IN and PEP individually or in-combination with other drugs. Most studies involved simultaneous analysis of only PEP and IN or AM and IN in a combined dosage form by HPLC [21, 22, 23,24].The present study sought to develop a HPLC analysis method for simultaneous determination of AM, IN and PEP in a single tablet formulation. We do acknowledge that El-Bagary et al[25] have conducted a similar experimentation, however, we created and validated a new methodology that aimed to enhance the analytical performance and improve the chromatographic separation of AM, IN and PEP when compared with El-Bagary et al study.

a.



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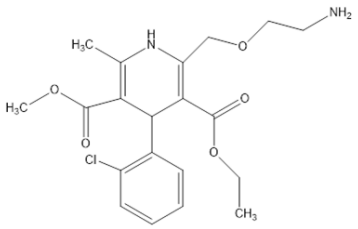

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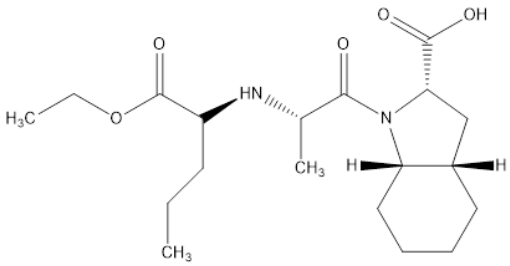


Fig.1: Chemical structures of (a) Indapamide, (b) Amlodipine and (c) Perindopril

2. EXPERIMENTAL

Reagent and materials

Indapamide, Amlodipine Besylate, and Perindopril Arginine raw materials were certified to have a purity of 99.90%, 99.85%, and 99.95%, supplied by Dishman (Ahmedabad, India), Aurobindo pharma (Hyderabad, India), and Aatri (Mumbai, India) respectively. Acetonitrile (HPLC grade) and Nylon Filter membranes (diameter = 47 mm, pore size = 0.45 µm) were obtained from Merck (Germany), trimethylamine (TEA) was purchased from Fisher (U.K). Phosphoric acid 85 % was supplied from Panreac (Spain). Combined tablets containing 3.7 mg of Perindopril Arginine (equivalent to 2.5 mg of PEP), 13.1 mg of Amlodipine Besylate (equivalent to 10 mg AM), and IN (10 mg) were formulated in the department of research and development, Dar Al Dawa, Jordan; as per requested. The approximate weight of the formulated tablet was 300 mg, and the mass percent composition was 7.4 % of active ingredients and 92.6% of expedients.

Instrumentation

The analysis method was developed and validated on HPLC Thermo- Scientific Dionex- Ulti- Mate 3000 system containing a gradient pump, UV-VISIBLE detector, column oven and a manual injector (Rheodyne with a 20 µL sample loop). The computational analysis was performed by Chromeleon 7 software. Specificity test was performed on water HPLC system consisting of Waters Alliance 2695 Pump, 2998 photodiode array detector (PDA) (Waters, Milford, MA, USA), Waters column heater and a Rheodyne injector with a 20 µL loop. Empower. ®. 3 software (Database Version 7.21.00.00) was used to process the data.

Chromatographic conditions

An Inertsil C8 column ((25cm x 4.6 mm, 5µm) GL Science, Japan) was used as the stationary phase. Isocratic elution was performed with a mobile phase composed of 0.7% triethylamine (TEA)(that was

dissolved in water and adjusted to pH 3 with orthophosphoric acid 85%) and mixed with acetonitrile in a ratio of 2:1(v/v). The mobile phase was filtered through a 0.45 µm membrane filter and degassed for at least 10 minutes before use. The flow rate was 1.5 mL/min. Detection was conducted at 205 nm with a UV–visible detector and at 200 to 260 nm with PDA detector.

Preparation of standard solutions

Three stock solutions with a concentration of 1000 µg/mL were prepared by separately weighing a 73.7 mg of Perindopril Arginine (equivalent to 50.0 mg PEP) , 69.5 mg of Amlodipine Besylate (equivalent to 50.0 mg of AM), and 50.0 mg of IN, and then transferring them into three separate 50 mL volumetric flasks that were finally dissolved in Acetonitrile. All stock solutions were kept at 4 °C and allowed to reach room temperature before use.

Preparation of sample solution

A quantity of powdered tablets of 480, 600, 720 mg that was equivalent to (4, 5,6) mg of IN, (16, 20,24) mg of AM and(16, 20,24) mg of PEP, were weighed and placed into 200 mL volumetric flasks. Around thirty milliliters of the mobile phase were added to the flasks and the solutions were placed in ultrasonic bath for 30 minutes to be dissolved. After that, the sample solutions were allowed to reach room temperature and the mobile phase was added to complete volumes. The solutions were filtered through Whatman filter papers No. 41 and 0.45 µm nylon filtration membranes. The obtained final concentrations were 20, 25, 30 µg/mL of IN, 80, 100, 120 µg/mL of AM, and 80, 100, 120 µg/mL of PEP.

Method validation

The developed method was subjected to validation test obtained from the International Conference of Harmonization (ICH) Guidelines for Validation of Analytical Methods[27]. Many parameters were determined, among which, system suitability test, linearity, specificity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

A system suitability test was performed by repeating injection of a standard solution (25 µg/mL of IN, 100 µg/mL of AM, and 100µg/mL of PEP) for ten times. A number of measurements including: peak shape, peak resolution, capacity factor (K'), and theoretical plate number (N) were carried out.

Stock solutions (1000 µg/mL) of IN, AM and PEP were mixed, diluted to various concentrations by adding different volumes of the mobile phase. Concentrations of the prepared solutions were: 20-30 µg/mL for IN, 80 – 120µg/mL for AM, and 80 –120µg/mL for PEP. Concentration ranges of the prepared solutions were equivalent to IN, AM, and PEP in the formulated drug samples (within range or at the extremes). These prepared solutions were used to study linearity and create calibration curves. An aliquot (20 µL) of each ternary mixture solutions was injected three times under the optimized chromatographic conditions and responses were collected by calculating the average peak area of the repeated injections. Calibration curves were made by plotting peak areas versus the concentrations and the correlation coefficients (R^2) were determined.

The percentage recoveries were calculated for AM, IN and PEP by three different concentration levels in order to evaluate the accuracy of the method. Standard solutions of IN (20, 25, and30 µg/mL), AM (80, 100 and 120µg/mL) and PEP (80, 100 and 120 µg/mL) were deliberately added to many placebo samples. Then those components were extracted and the main recovery was calculated using the following equation:

$$\text{Recovery \%} = \frac{\text{amount found in the samples}}{\text{Spiked amount}} X\ 100\%$$

The intra-day (same day) and inter-day (different days) precision of the suggested analytical method were evaluated by recording three responses for each concentration level on the same day and three responses for each concentration level on three different days, and the concentration levels used were 25 µg/mL for IN, 100 µg/mL for PEP and AM. The precision, which is usually

expressed as relative standard deviation (RSD), was required to be less than 2%.

Repeatability was evaluated by recording response ten times at one concentration level. It was performed on 25 µg/mL for IN and concentrations of 100 µg/mL for AM and PEP.

The LOD and LOQ were calculated for this method to evaluate its sensitivity. The LOD and LOQ were detected by using the following mathematical equations[27]:

LOD = 3.3x σ_{n-1}/m,

LOQ =10 x σ_{n-1}/ m

where σ_{n-1} is standard deviation of the blank and m is the slope of regression equation.

Specificity test was performed by HPLC equipped with PDA detector to determine the purity of the peaks obtained for IN, AM, and PEP. A standard of PEP (100 µg/mL), AM (100 µg/mL), IN (25 µg/mL) and placebo sample (3000 µg/mL) were analyzed. The obtained chromatographic peaks for all components were investigated for peak purity by comparing the spectra at three specific regions: peak start, peak apex and peak end. The used scanning range was 200 –260 nm.

Robustness of the methods was tested. The same standard mixture (containing 25 µg/mL IN, 100 µg/mL AM and 100 µg/ml PEP) was reanalysed by HPLC for several times after intended alteration in the method parameters. Changes in the responses of AM, PEP and IN were recorded. Method parameters were altered including: the mobile phase pH (∓0.1), column oven temperature (± 2° C), mobile phase composition (± 3% of acetonitrile), the flow rate (∓0.2 mL), and the detection wavelength (∓ 2 nm). The precision (RSD) was calculated and investigated if it was less than 2%.

3. RESULTS AND DISCUSSION

A system suitability test for HPLC was assessed before measuring other validation parameters and running quantitative analysis. The responses were recorded for ten replicate injections of the prepared standard with a concentration of 25 µg/mL IN, 100 µg/mL AM and 100 µg/mL PEP. Several parameters were evaluated for the optimized HPLC method, among which the capacity factor, the peak resolution (K'), asymmetry of the peak, theoretical Plate (N), selectivity factor, and RSD of the peak area (Table 1).

Table 1. System Suitability Parameters for the HPLC Method

Parameter	PEP	AM	IN
Capacity Factor	2.69	4.88	8.72
Resolution Factor	3.47	10.5	-
Asymmetry Factor	1.0	1.3	1.2
Theoretical Plate	380	4776	3265
Selectivity Factor	1.81	1.79	-
RSD of the peak area (%)	0.28	0.03	0.14

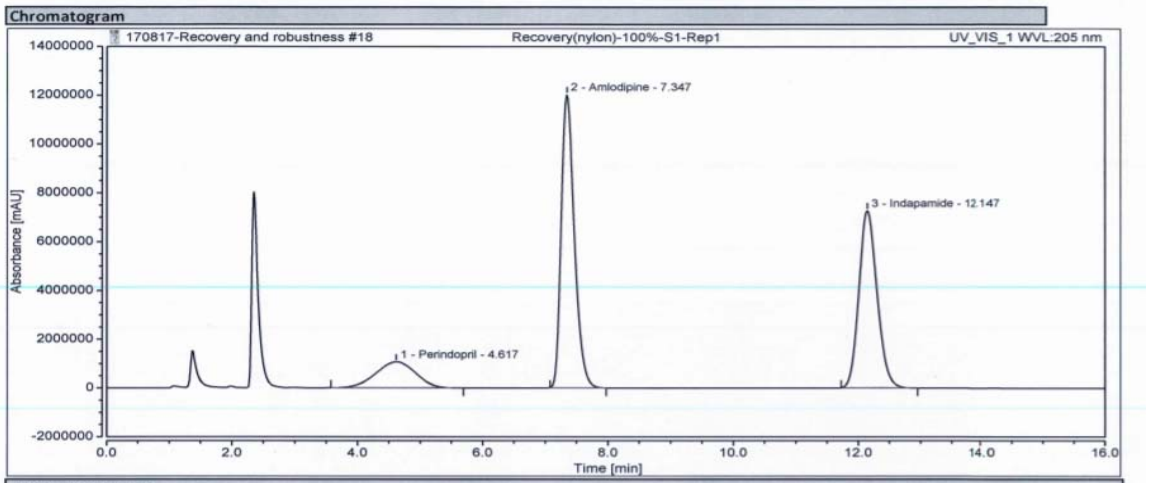
The capacity factor values were estimated at 2.69, 4.88 and 8.72 for PEP, AM, IN, respectively. Compared to El-Bagary et al study, the values of capacity factors were estimated at 0.77, 2.05 and 4.38 for PEP, AM, IN, respectively. This method showed a good improvement in the capacity factor of PEP. The resultant small capacity factor for PEP (0.77) highlights a short interaction time between PEP and the stationary phase[26].

The estimated resolution factors were 3.47 and 10.5 which indicated that all components were totally separated and not eluted together. One of the estimated resolution factors in our study is higher than that reported by El-Bagary et al (3.37 and 4.59)[25]. The reported values of asymmetry factors (1.0, 1.1 and 1.3) confirmed that all peaks were symmetrical[28]. These reported values of asymmetry factors are in line with those reported in the El-Bagary et al study. Theoretical plates were estimated at 380, 4776, and 3265 for PEP, AM, IN, respectively. The theoretical plate values revealed an excellent chromatographic performance for both AM and IN and not for PEP which exhibited a reduced efficiency of

separation. Comparing theoretical plate values with that reported from El-Bagary et al study. We found the efficiency of separation for PEP to be lower by 0.27 times compared to that reported by El-Bagary et al whereas theoretical plate's values for AM and IN were higher by 1.5 and 1.3 times respectively compared to that reported in the El-Bagary et al study. Moreover, PEP owned the broadest peak due to its high band diffusion[26]. The estimated selectivity coefficients in this method were 1.81 and 1.79. Selectivity factors were reported by El-Bagay et al as 2.66 and 2.14. Both of the two methods showed high selectivity and absence of coeluting[28]. The percent relative standard deviation (%RSD) of the peak area responses were 0.28% for PEP, 0.03% for AM, and 0.14% for IN in this study. El-Bagary et al reported the percent relative standard deviation (%RSD) of the peak area responses as 0.77% for PEP, 0.58% for AM, and 0.29% for IN.

The mobile phase was selected after testing several mobile phases to achieve optimized HPLC parameters, such as reasonable run time, absence of noisy lines, sharp peaks with high resolution and acceptable symmetry. Perindopril arginine was hydrolysed to arginine and PEP. As shown in Figure 2, four symmetrical peaks with good separation were obtained on a C 8 column through use of a mobile phase composed of 0.7% TEA solution (its pH was adjusted to 3.0 with 85 % H_3PO_4) and acetonitrile in a ratio of 2:1(v/v). The obtained retention times were 2.4 minutes for Arginine, 4.6 minutes for PEP, 7.3 minutes for AM, and 12.1 minutes for IN when analysis was performed at a flow rate of 1.5 mL/min and detection wavelength of 205 nm as shown in Figure 2.

The suggested analytical method was validated in obedience with the International Conference on Harmonization guidelines (ICH). Results of the various parameters are presented in Table 2.



Integration Results

No.	Peak Name	Retention Time	Area	Asymmetry (EP)	Resolution (USP)	K'	Peak Type	Rel Area
		min	mAU*sec					%
1	Perindopril	4.617	786900	1.0	3.47	2.69	Autodetect	12.77
2	Amlodipine	7.347	2924017	1.3	10.50	4.88	Autodetect	47.46
3	Indapamide	12.147	2450432	1.2	n.a.	8.72	Autodetect	39.77
Total:			**6161349.374**	**3.46**	**13.97**			

Fig 2: HPLC chromatogram of a standard mixture of PEP (100 µg/mL), AM (97 µg/mL), and IN (25 µg/mL).

Three linear calibration curves were obtained after plotting peak area versus concentration for IN, AM, and PEP in the ranges of 20–30, 80–120, and 80–120 µg/mL, respectively (Table 2). The obtained linear equations were: Y = 97468 x+ 28462, Y = 30756 x -53413, and Y = 6927 x + 95761 for IN, AM and PEP, respectively. El-

Bagary et al reported concentration ranges: 5-20 µg/mL for IN, 2.5-80 µg/mL for AM, and 5-80µg/mL for PEP. The obtained linear equations from El-Bagary et al study were Y = 1.3587x− 0.1295 for IN, Y = 0.4718 x -0.0081 for AM, and Y = 0.1623x + 0.0288 for PEP. Slopes values are higher in our study than those obtained from El-Bagary et al study and this confirms that the chromatographic conditions and concentration ranges in our study participate in having better instrumental response toward IN, AM and PEP. The correlation coefficient (R^2) values were 0.998 for IN, 1.00 for AM and 0.995 for PEP. The LOD detected values were 0.99, 0.38 and 3.56 µg/mL for AM, IN and PEP, respectively. The LOQ detected values were 3.01, 1.16 and 11.06 µg/mL for AM, IN and PEP, respectively. IN owned the lowest level of LOD and LOQ as shown in the table 2.

The percentage recovery values of IN, AM, and PEP were extended from 99.9 to 101.4%. These values agreed with those reported by El-Bagary et al. High values of recovery with RSD values less than 1% for all drugs at different concentration confirms the accuracy of this analysis method. The values of the accuracy studies are summarized in Tables 2.

The obtained results from Inter-day and intra-day precision test showed that the RSD values were less than 2% for all injected samples. Low obtained values of RSD can be considered a confirmation to that the analysis method is precise and repeatable. The precision values are summarized in table 2.

Table 2. Summary of Validation Parameters for the Proposed Method

Parameter	PEP	AM	IN
Range of linearity (µg/mL) n =3	80–120	80–120	20–30
Regression equation	Y = 6927 x+ 95761	Y=30756 x -53413	Y = 97468 x + 28462
Correlation Coefficient R^2	0.995	1.000	0.998
Inter-day precision (RSD %) n=6	0.56	0.43	0.12
Intra-day precision (RSD %) n=6	0.45-1.0	0.25-0.99	0.49-0.98
Specificity	Specific	Specific	Specific
Repeatability (RSD %) n=10	0.14	0.04	0.24
LOD (µg/mL)	3.65	0.99	0.38
LOQ (µg/mL)	11.06	3.01	1.16
Recovery (%)±SD (n= 3)	99.92±0.39-100.02± 0.64	100.5±0.04-100.7±0.13	100.9±0.1- 101.4±0.08

The peak purity of AM, IN and PEP was investigated through comparing their respective spectra at three regions: peak start, apex and peak end. The peak purity value for all three drugs exceed 990 (ideal value, 1,000), which means that the peaks were pure and free from interfering peaks. Therefore, the proposed analysis method was specific for the three drugs and the excipients had no effect on the separation process, retention times, shape and width of the peaks.

The robustness of the method was studied by achieving assays of a standard mixture containing 20 µg/mL of IN, 100 µg/mL of AM and 100 of µg/mL PEP.

The parameters of the method were purposely changed, and alterations in the responses of AM, IN and PEP were recorded. The assay values of the three drugs were calculated in the changed parameters. Changes of the different parameters did not highly affect the retention times and peak areas. The proposed method proved to be robust, because these minor changes in flow rate, pH, and wavelength, mobile phase composition and column oven temperature do not significantly affect the obtained results. The RSD was less than 2% under different experimental conditions as shown in Table 3.

Table 3. Robustness test of IN, AM, and PEP by the HPLC Method

Altered Parameter	IN(assay ± SD)	AM(assay ± SD)	PEP(assay ± SD)
Acetonitrile composition (±3%)	100.8 ± 0.3	100.4 ± 0.2	99.6 ± 0.4
pH(±0.10)	100.3 ± 0.1	100.2 ± 0.2	99.8 ± 0.3
Wavelength (±2 nm)	100.4 ± 0.2	100.6 ± 0.4	99.0 ±1.0
Column oven temperature (± 2°C)	100.5 ± 0.5	100.6 ± 0.4	98.3 ±1.0
Flow Rate (±0.2 mL/min)	100.3 ±0.2	100.6 ± 0.3	99.2 ±0.6

4. Conclusion

This study shows that the developed high performance liquid chromatographic method was applied successfully to simultaneously determine Indapamide, Amlodipine Besylate and Perindopril Arginine in a combined single formulated tablet. Validation data studies showed that the method was precise, sensitive, selective, robust, and linear over the concentration range of 20-30 µg/mL for Indapamide, and 80-120 µg/mL for Amlodipine Besylate and Perindopril Arginine. This method was also free from interference from the excipients used in the formulations. This method showed improvement in capacity factor of Perindopril Arginine and theoretical plates for Amlodipine Besylate and Indapamide when compared to the previously reported study. The main advantages of this method were rapidness and easiness. These results allowed us to conclude that the developed method can be used in routine analysis.

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التحديد المتزامن للانداباماید والأملودیبین والبیرندوبریل في الأقراص باستخدام الفصل الكروماتوغرافي عالي الكفاءة

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ملخص

أجريت هذه الدراسة لتطوير طريقة تحليل دقيقة للتحديد المتزامن للانداباماید والأملودیبین والبیرندوبریل في الأقراص و التثبت منها باستخدام الفصل الكروماتوغرافي عالي الكفاءة. تم تنفيذ الفصل الكروماتوغرافي باستخدام عامود فصل من نوع (Inertsil C8) أبعاده (25 سنتيمتر*4.6 ملمتر*5 ميكرومتر) محفوظ في درجة حرارة 40°س, و طور متحرك يتكون من خليط محلول ثلاثي ايثيل الامين المنظم (رقمه الهيدروجيني 3.0) و الاسيتونايتريل بنسبة (2:1) بمعدل تدفق 1.5 مللتر/دقيقة و على طول موجي 205 نانومتر للكشف, بحيث ينتهي فصل المواد الثلاثة في أقل من 20 قيقة. تم التثبت من طريقة التحليل استناداً إلى المبادئ التوجيهية للمؤتمر الدولي للتنسيق. من خلال هذه الدراسة يمكننا التوصية باستخدام طريقة التحليل هذه في كافة مختبرات تحليل الأدوية لتحليل الانداباماید والأملودیبین والبیرندوبریل.

الكلمات الدالة: .التحديد المتزامن،انداباماید، الأملودیبین والبیرندوبریل،استخدام الفصل الكروماتوغرافي عالي الكفاءة

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Pharmacognostical and biological exploration of *Scaevola taccada* (Gaertn.) Roxb. grown in Egypt

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ABSTRACT

Identification criteria and quality control of *Scaevola taccada* (Gaertn.) Roxb. cultivated in Egypt were scanty. The present study aims to appraise characters of *S.taccada* grown in Egypt, as a traditional medicinal plant. A detailed botanical study of leaf, stem, and flower of *S. taccada* were investigated to find out their characters in entire and powdered form. DNA fingerprinting of leaves was carried out using two polymerase chain reaction (PCR) dependent techniques. Total carbohydrate, lipid and protein content were estimated. Lipoids were subjected to gas liquid chromatography (GLC). Spectrophotometric analysis of polyphenolic contents was conceded out. Comparing the results of the two PCR-dependent techniques revealed that inter-simple sequence repeat (ISSR) will be more useful and informative than random amplified polymorphic DNA (RAPD) in identification of *S.taccada*. Proximate analysis of dried leaf powder showed total, water soluble, acid insoluble ash and moisture content as 14.95%, 5.07%, 5.07% and 9.11w/w respectively. Nutritive value examination revealed a high protein 12% and appreciable carbohydrates contents. *n*-pentacosane 27.70%, stigmasterol 13.16% and α-linolenic acid 12.19% were detected as major lipoids. The antihyperglycemic and the anti-inflammatory activities of the ethanolic and aqueous extracts of the leaves were carried out. Both the ethanolic and the aqueous extracts (200mg/kg) were exhibited noteworthy hypoglycemic effect alike to gliclazide (10 mg/kg). Furthermore, the ethanolic extract was prevailed a parallel decrease in the cholesterol and triglycerides level in streptozotocin (STZ)-Induced Rat Diabetic Model. Moreover, the ethanolic extract (100mg/kg) evidence a significant anti-inflammatory effect 87.17 and 91.88% for 3, 4h treatment respectively compared to indomethacin.

Keywords: *Scaevola taccada,* Goodeniaceae, macromorphology, micromorphology, DNA fingerprint, antihyperglycemic, anti-inflammatory.

1. INTRODUCTION

Correct characterization and quality assurance of starting material is an essential step to ensure reproducible quality of herbal medicine which will help us to justify its safety and efficacy.(1) Intended for this purpose we have done a pharmacognostical and biological studies of *Scaevola taccada* (Gaertn.) Roxb. family Goodeniaceae grown in Egypt. Goodeniaceae is a family of flowering plants included within the genus Asterales and considered as a sister group to family Asteraceae.(2) It comprises 11 genera and approximately 400 species.(3) Most Goodeniaceae genera are indigenous to Australia, one genus; *Scaevola* is mainly dispersed throughout the pacific area. (4)Members of the family are either herbs or shrubs.(5)The largest genera are *Goodenia*, *Scaevola* and *Damperia.*(6) Family Goodeniaceae is characterized by the presence of some important phytoconstituents: coumarins, (7) iridoid glycosides, (8) pentacyclic triterpenoids: myricadiol and taraxerol,(9) betulin and betulinic acid (10) and inulin.(11) Based on recent researches, these compounds were found to exhibit

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various biological activities of medical importance to treat many diseases.[12]

Scaevola comprises about 130 species, among these 40 are growing outside Australia and two species are widely distributed throughout the Pacific and Indian oceans [4,9] reported that genus *Scaevola* has been used for treatment of diabetes. The name "*Scaevola*" is derived from the Greek word "*Scaevus*" meaning "left-handed[13], also "*Scaevola* " means "little hand."[3] Both names refer to the shape of the flowers which having petals directed to one side giving hand or fan-shaped flowers.

Scaevola taccada (Gaertn.) Roxb. is known as *S. frutescens* (Mill) Krause*, S. plumieri, S. lobelia*[14] and *S.sericea*.[15] It is widely distributed along the coasts of Africa, Indian oceans, tropical Australia and through the pacific oceans from Taiwan to the Hawaiian islands.[4]

Traditionally, different parts of *S.taccada* were used for treatment of various ailments. Leaves were reported to treat indigestion and also used as poultice for headache.[16] The crushed fruits have been used to treat tinea.[15] Medicinally, leaves have been reported to act as anti-diabetic, antipyretic, anti-inflammatory, anticoagulant, skeletal muscle relaxant and also as antimicrobial agent.[17] *S. taccada* is reported to contain phenolic compounds, proteins and carbohydrates, while alkaloids and saponins were completely absent. [18]

DNA-based tools for authentication of medicinal plants is utilized in any form of the drug processed or unprocessed.[19] Random amplified polymorphic DNA (RAPD) has been widely used for the authentication of plant species of medicinal importance. The use of ISSR markers for assessing genetic purity has been reported in agricultural crops like rice, [20] sunflower,[21] maize. [22] By reviewing the current literature, no data was reported concerning the botanical and biological features of *S. taccada* grown in Egypt. Therefore, the main objective was to scrutinize the botanical and DNA profiling as well as corroborate its traditional uses on experimental basis.

Methodology

Plant material

Samples of *S. taccada* (leaves, stems &flowers) used in this study, were collected during the years 2013-2014 from private garden, Giza, Egypt which is specialized in cultivation and spreading of the medicinal plant. The plant material was kindly identified by Agricultural Engineer Therese Labib**.,** Consultant of Plant Taxonomy at Ministry of Agriculture and the Former Director of Orman Botanical Garden, Giza, Egypt. A voucher specimen (28-6-2016) is kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy Cairo University.

Botanical profiling

Specimens of fresh samples of the plant organs under investigation, as well as, samples kept in ethanol (90%) containing glycerin (5%) were used for both morphological and histological studies. Leica light microscope equipped with Leica Queen 550IW digital camera: Leica microsystems, (Wetzlar, Germany) utilized for anatomical examinations. Results were depicted in (Table 1 and Figures1-5).

Table 1: The microscopical measurements of the elements of different organs of *S. taccada*(Gaertn.)Roxb. (Measured in microns)

Element	Length	Width	Diameter	Height
A- The Stem				
Cork	33-50-75	33-37-50		14-21-28
Epidermal cells	35-42-57	28-57-64		8-13-16
Calcium oxalate clusters			9-11-14	
Calcium oxalate prisms	25-35-40	15-20-31		
Pericyclicfibres	305-450-950	8-14-18		

Wood fibres	410-550-750	23-25-38		
Wood parenchyma	85-95-105	22-27-30		
Xylem vessels			11-15-35	
Medullary rays	55-65-75	50-60-65		
Starch granules			8-12-17	
B- The leaf				
Upper epidermal cells	50-60-70	35-45-50		16-20-25
Lower epidermal cells	15-30-60	18-24-42		8-16-25
Neural epidermal cells	24-46-60	19-21-23		9-12-15
Petiole epidermal cells	44-62-106	16-19-22		3-5-6
Stomata	27-30-33	21-24-31		
Calcium oxalate prisms	13-14-18	12-13-14		
Calcium oxalate clusters			6-8-10	
Calcium oxalate rosettes			33-30-42	
Medullary ray cells	60-90-120	39-60-66		
Wood fibers	380-425-470	4-6-11		
Wood parenchyma	165-180-200	30-45-60		
Xylem vessels			12-14-25	
Tracheides	295-318-340	54-63-68		
Starch granules			12-20-23	
Non-glandular trichomes	250-275-287	6-13-19		
C-The flower				
1-Calyx				
Outer epidermal cells	25-35-55	20-25-30		6-9-13
Inner epidermal cells	29-42-57	25-34-40		10-15-20
Stomata	25-30-40	22-25-30		
Non-glandular trichomes	170-175-190	21-23-26		
Xylem vessels			25-29-33	
Tracheids	130-152-161	17-30-35		
Calcium oxalate clusters				7-10-12
2-Corolla				
Outer epidermal cells (apical)	28-40-55	20-28-33		
Outer epidermal cells (middle)	26-28-35	18-23-23		
Outer epidermal cells (basal)	46-60-78	25-30-39		
Inner epidermal cells (apical)	18-45-68	18-31-41		
Inner epidermal cells (middle)	175-190-250	15-30-45		
Inner epidermal cells (basal)	32-41-68	14-23-25		
Stomata	32-34-35	26-27-28		
Xylem vessels			19-22-33	
Non- glandular trichomes	30-33-35	9-18-22		
3- Androecium :				
Filament epidermal cells	143-157-207	21-25-29		

Anther epidermal cells	21-29-33	23-29-32		
Fibrous layer of anther	25-41-62	19-22-25		
Pollen grains			36-60-71	
Xylem vessels			25-29-33	
Calcium oxalate rosette				
4-Gynaecium				
Ovary epidermal cells	23-29-38	23-29-35		1-3-5
Style epidermal cells	32-43-50	12-14-16		
Calcium oxalate rosettes			3-6-7	
Xylem vessels			14-17-18	
Non-glandular trichomes	77-116-125	11-14-16		
5-Pedicel				
Epidermal cells	31-42-65	17-23-30		8-13-17
Non-glandular trichomes	91-95-98	9-10-12		
Prism of calcium oxalate	20-30-35	13-20-29		
Xylem vessels			8-11-27	

Materials for DNA mapping

Buffers: The following buffers were used: *Extraction buffer*: 1.4 M NaCl, 0.1 M Tris (pH 7.5), 20 mM EDTA, 2%(w/v)N-cetyl-N,N,N-tri-methyl ammonium bromide(CTAB), 1% (v/v) β-mercaptoethanol (added immediately before use); *Washing buffer*: 1:76% ethanol, 0.2 M Na-acetate; 2:76% ethanol as washing buffer, 10 mM NH_4 O-acetate, TE-buffer;10 mMtris-HCl(pH 8.0), 1mM EDTA,10x; *Reaction buffer*: 100 mMtris-HCl (pH 8.3), 500 mMKCl, 0.01% (w/v) gelatin, chloroform/ isoamyl alcohol 24:1 (v/v), isopropanol, d NTP, Taq DNA polymerase.

Primers: RAPD & ISSR practice were carried out in triplicates by using genomic DNA with 11 decamer primers for reproducibility of the consequences. Six primers were used for the RAPD analysis with the following sequences: OPA-01: 5′CAG GCC CTT C 3, OPA-07: 5′ GAA AGG GGT G 3′, OPA-10: 5′GTA GAC CCG T 3′, OPB-01: 5′GTT TCG CTC C 3′, OPB-07:5′ GGT GAC GCA G 3′, OPM-01:5′ ACG GCG TAT G 3′. Five primers were used for the ISSR analysis with the following sequences: HB-08: 5′ GAG AGA GAG AGA GG 3′, HB-10: 5′ GAG AGA GAG AGA CC 3′, HB-11: 5′GTG TGT GTG TGT TGT CC 3′, HB-13: 5′GAG GAG GAG GC 3′, HB-14: 5′CTC CTC CTC GC 3.

Molecular weight markers: 100bpladder (New England Biolab Co., UK.)

Equipment: DNA Thermal Cycler, (Perkin Elmer, TA. Warrington, UK) for amplification of DNA, agarose gel electrophoresis tool, for separation of RAPD fragments according to size (Gibco BRL Life Technologies, Paisley, UK) and UV Polaroid camera used for) for visualization of fragments.

Methods for molecular investigations

DNA extraction:

DNA analysis was conducted at Food Technology Research Institute, Agriculture Research center, Ministry of Agriculture and Land Reclamation, Giza, Egypt in 2016. DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) method.[23] Fifty mg of frozen leaf were pulverized in liquid nitrogen, extracted with 0.8 ml CTAB and precipitated with isopropanol.

Assessment of DNA planning:

DNA concentration was determined by diluting the DNA 1:5 in distilled H_2O. The DNA samples were electrophoresed in 1% agarose gel against 10 µg of a DNA size marker. This marker covers a range of concentration between 95 ng and 11 ng. Thus, valuation

of the DNA concentration in a prearranged sample was achieved by comparing the intensity of fluorescence of the unknown DNA band with the dissimilar bands in the DNA size marker.

Magnification of RAPD, ISSR markers:

The PCRs were conceded out using 100 ng of genomic DNA template subsequent a thermal cyclic program. (24)

Thermocyling Profile:

Magnification of PCR was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (*PE* Applied Biosystems, USA) automatic to accomplish 35 cycles later than an early denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 45ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer expansion segment was wholesale to 7 min at 72ºC in the closing cycle. The augmentation products were determined by electrophoresis in a 1.5 % agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. A 1kb DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (Bio-Rad 2000, Germany).

Data analysis

The banding patterns generated by RAPD, ISSR-PCR marker analyses were scored as present (1) or absent (0), each of which was treated as a sovereign character in spite of its intensity (Fig.6). Only major and reproducible bands obtained for each RAPD, ISSR primer were measured. By comparing the banding patterns of species for a primer, species-specific bands were identified. Faint or indistinct bands were not measured.

Determination of Proximate and Macronutrient Composition

Proximate analysis included the determination of certain analytical standards (ash value and moisture content), as well as that of macronutrient (i.e. total or crude carbohydrate, lipid and protein) contents according to published procedures. (25,26)

Investigation of the Lipoidal Contents

Gas chromatographic profiling of the lipoids

One gram of the petroleum ether fraction of the leaves of *S.taccada* was subjected to saponification. The unsaponifiable and saponifiable lipoids were prepared from the petroleum ether extract (PE) of the leaves of *S. taccada* subjected to gas liquid chromatography (GLC).

Preparation of the unsaponifiable matter

The unsaponifiable matter (USM) was prepared from the petroleum ether extract (PE, 1.0 g) according to Vogel (1975).(27) The solvent-free residue (0.30g), representing the USM, was saved for further GLC analysis. The aqueous alkaline solution, left after separation of the USM, was acidified with dilute hydrochloric acid (5N) to liberate the free fatty acids (FA). These were extracted with diethyl ether (4×50mL). The extract was then dehydrated over anhydrous sodium sulphate and the solvent evaporated to dryness yielding a 0.60g residue representing the free FA.(27)

Preparation of the fatty acid methyl esters (FAME)

The FA mixture as well as the standard fatty acids was, separately, dissolved in small amounts of anhydrous methanol.(28) After 10 min, the solvent was evaporated at room temperature under a stream of nitrogen and the dried residue saved for GLC analysis.

GLC analysis of the unsaponifiable matter (USM)

USM was subjected to GLC on Hewlett-Packard HP-5890 N system equipped with an FID detector,280°C; air flow rate: 350ml/min and H2 flow rate 50ml/min. Analysis was performed on a ThermoTR-5-MS coated with 5% phenyl polysilphenylene siloxane column (30mx0.25mmx0.25µmfilm thickness); injector temperature 270°C, using N_2 as carrier gas and adopting a temperature programming as initial temperature, 70°C,kept isothermal for 2 min, increased to 280°C by the rate of 5°C/min, then kept isothermal. Flow rate 30ml/min. Aliquots,2 µL each, of 2% chloroformic solutions of the USM and reference samples were co-

chromatographed. Identification of the component hydrocarbons, phytosterols and triterpenoids was based on comparison of the retention times observed for the different peaks in the GLC chromatogram of the sample to those of the available authentic samples. The relative amount of each component was calculated *via* peak area measurement using a computing integrator (Table 2).

Table 2: Components identified by GLC analysis of the USM of the leaves of *S. taccada* (Gaertn.) Roxb.

No.	RR_t*	Carbon no.	Identified component	Percentage
1	0.37	C_{14}	n-Tetradecane	1.16
2	0.47	C_{15}	n-Pentadecane	1.59
3	0.49	C_{16}	n-Hexadecane	2.5
4	0.56	C_{17}	n- Heptadecane	2.18
5	0.61	C_{18}	n-Octadecane	13.42
6	0.64	C_{19}	n-Nonadecane	1.93
7	0.72	C_{20}	n-Eicosane	1.29
8	0.76	C_{21}	n-Heneicosane	1.09
9	0.83	C_{22}	n-Docosane	2.66
10	0.87	C_{23}	n-Tricosane	0.73
11	0.95	C_{24}	n-Tetracosane	2.13
12	1	C_{25}	n-Pentacosane	27.70
13	1.06	C_{27}	n-Heptacosane	2.02
14	1.13	C_{29}	n-Nonacosane	3.89
15	1.15	C_{27}	Cholesterol	3.27
16	1.2	C_{28}	Campesterol	2.98
17	1.25	C_{29}	Stigmasterol	13.17
18	1.31	C_{29}	*β*-Sitosterol	6.1
19	1.5	C_{30}	α-Amyrin	2.6
Total identified components				92.41%
Identified hydrocarbons				64.29%
Identified phytosterols				25.52%

*RR_t= Retention time relative to *n*-pentacosane (R_t=22.12 min).

GLC analysis of the fatty acid methyl esters (FAME)

FAME sample was analyzed using GLC Trace GC Ultra system equipped with a FID detector. Analysis was performed using a Thermo TR-FAME column (70%Cyanopropyl Polysilphenylene Siloxane) (30mx 0.25mmx 0.25µm film thickness); injector temperature

200°C, using N_2 as carrier gas and adopting a temperature programming as initial temperature, 140°C, increased to 200°C by the rate of 5°C/min, then kept isothermal for 3min. Flow rate 30ml/min. with N_2 as carrier gas. Aliquots, 2 µL each, of 2% chloroformic solutions of the analyzed FAME and reference fatty acid methyl esters were analyzed under the same conditions. The amounts of individual FA were computed as mentioned under the USM. Identification of the hydrocarbons and sterols was based on comparing the retention time of their peaks with those of the available reference standards. The amount of each component was calculated by peak area measurement using a computing integrator (Table 3).

Table 3: Components identified by GLC analysis of the FAME of the leaves of *S. taccada*(Gaertn.)Roxb.

No.	RR_t*	Carbon no.	Fatty acids corresponding to FAME	Percentage
1	0.55	C_{12}	Lauric acid	0.90
2	0.78	C14	Myristic acid	7.64
4	0.89	C15	Pentadecanoic acid	0.56
5	1	C16	Palmitic acid	34.60
6	1.096	C17	Margaric acid	0.92
7	1.195	C_{18}	Stearic acid	6.19
8	1.22	C(18:1)	Vaccenic acid	7.38
9	1.228	C(18:1)	Oleic acid	0.4
10	1.27	C(18:2)	Linoleic acid (Omega-6)	7.78
11	1.34	$C_{(18:3)}$	Linolenic acid (Omega-3)	12.19
12	1.37	C_{19}	Nonadecanoic acid	6.15
13	1.46	C_{21}	Heneicosanoic acid	0.7
14	1.54	C_{22}	Docosanoic acid	4.55
15	1.62	C_{23}	Tricosanoic acid	1.29
16	1.70	C_{24}	Tetradecanoic acid	1.90
Total identified components				93.15%
Saturated fatty acids				65.4%
Unsaturated fatty acid				27.75%

*RR_t: Relative retention time relative to palmitic acid,(Rt =23.79 min.)

Spectrophotometric determination of phenolic and flavonoid contents

The total phenolic and flavonoid contents were determined in the leaves of *S. taccada* according to published spectrophotometric procedures. [29-31]

Determination of total phenolic content

Spectrophotometric determination of total phenolic content (TPC) was carried out by the Folin-Ciocalteu colorimetric method, as described in the European Pharmacopeia, [30] and modified by Ivanova et al., (2010).[31] The total phenolic content was expressed as Gallic acid equivalents (mg GAE/100mg extract) and

deduced from the pre-established calibration curve. Triplicate experiments were carried out for each sample.

Determination of total flavonoid content

Colorimetric method was adopted, based on measuring the intensity of the color developed when flavonoids are complexes with aluminum chloride method. [29,32] The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per 100 mg extract. Experiments were carried out in triplicates, and average absorbance values recorded.

In-vivo Biological study

Experimental animals

Adult male Wistar rats were obtained from the animal house colony, National Research Center, Giza, Egypt. Animals weighing 150-200 g were used for determination of LD_{50} and evaluation of antihyperglycemic and anti-inflammatory activities. Experimental animals were housed at a temperature of 23 ± 2ºC and 55 ± 5% humidity with 12 hr light/dark cycle, and free access to standard food pellets composed of vitamins mixture (1%), minerals mixture (4%), corn oil (10%), sucrose (20%), cellulose (0.2%), casein (10.5%) and starch (54.3%). Water was supplied *ad. libitum*. The experimental protocol followed was that of the Institutional Animal Ethical Committee of the National Research Centre.

Determination of Median Lethal Dose (LD_{50})

LD_{50} of the 90% ethanolic extract of the leaves of *S.taccada* was performed in accordance to Organization for Economic Co-operation and Development OECD-425 guidelines.[33] Five Wistar albino rats of uniform weight were selected; one of which was fasted overnight with free access to water. They were given 2000 mg/kg of the tested extract orally and were observed along 24 hrs for mortality. The animal survived and then four additional animals were tested sequentially so that a total of five animals were tested. All the animals were observed closely for 24 hrs daily for 14 days, no mortality was observed. Therefore a dose of 200 mg/kg ($1/10^{th}$ of 2000 mg/kg) was selected as the maximum safety dose. The ethanolic extract was considered safe up to a dose 2 g/kg b.wt.

Evaluation of the antihyperglycemic activity

The ethanolic and the aqueous extracts of the leaves of *S.taccada* were evaluated for their antihyperglycemic activities. Induction of diabetes mellitus was done by a single intraperitoneal (i.p.) injection of streptozotocin (STZ), 55 mg/kg b.wt., freshly prepared in 0.1 M citrate buffer (pH 4.5). [34] A normal control group (n = 6) was injected (i.p.) with the appropriate volume of the citrate buffer. After 48 hrs., blood samples were withdrawn from the retro-orbital venous plexus under light ether anesthesia, and the serum was separated by centrifugation for the determination of the glucose level. The rats having FBG values ≥ 230 mg/dl were selected and considered as hyperglycemic animals and were subjected to further experimentation. Diabetic rats were randomly divided into six groups (six rats each). The first group remained untreated during the whole study period. The second and third groups were administered orally with 100 and 200 mg/kg b.wt. of the ethanolic extract suspension in distilled water, respectively. The fourth and the fifth groups were supplied orally with 100 and 200 mg/kg b.wt. of the aqueous extract and the last group was treated orally with 10 mg/kg b.wt. gliclazide hydrochloride dissolved in distilled water (as standard antihyperglycemic drug). The animals received the indicated treatments every day for 2 weeks. At the end of 2-week treatment, the animals were kept for an overnight fasting and the blood samples were collected from retro-orbital plexus and allowed to clot for 30 min at room temperature. These blood samples were centrifuged at 5000 rpm for 20 min and the serum was obtained for determination of the serum glucose, cholesterol and triglycerides levels.

Determination of glucose level

Blood glucose level was estimated by a test reagent kit according to the method of Trinder, 1969. [35] The absorbance was measured at 510 nm and the results were expressed as mg/dl. Results are recorded in Table (4).

Table 4: Antihyperglycemic activity of the ethanolic and aqueous extracts of *S. taccada* leaves in STZ-induced diabetic rats.

Group	Glucose level (mg/dl)	Cholesterol level (mg/dl)	Triglyceride level (mg/dl)
Normal	86.08±3.76@	115.9±5.68@	106.6±7.76
Control (diabetic)	232.3±14.03*	159.8±11.14	132.0±10.68
Ethanolic extract 100 mg/kg	186.8±10.87* (19.5%)	136.9±5.034 (14.33%)	114.6±3.803 (13.18%)
Ethanolic extract 200 mg/kg	115.3±11.6@ (50.37 %)	120.3±6.671@ (24.71%)	108.0±9.312 (18%)
Aqueous extract 100 mg/kg	185.5±15.68* (20.1%)	141.0±5.341 (11.76%)	131.5±8.825 (0.37%)
Aqueous extract 200 mg/kg	110.2±8.93@ (52.56%)	138.2±6.322 (13.51%)	110.1±7.246 (16.59%)
Gliclazide10 mg/kg	101.7±4.53@ (56.22%)	105.6±9.086@ (33.91%)	106.9±7.846 (19.01%)

*Statistically significant from the normal group at $p<0.05$.

@Statistically significant from the control group at $p<0.05$

Statistical analysis was carried out using repeated one-way ANOVA test followed by Tukey test for multiple comparisons

Determination of serum triglyceride level

Triglycerides level was estimated by enzymatic methods using diagnostic kit according to the method of Fossati and Prencipe, 1982.[36]The absorbance was measured at 510 nm and the results were expressed as mg/dl. Results are presented in Table (4).

Determination of serum cholesterol level

Cholesterol level was estimated by enzymatic methods by using diagnostic kit according to the method of Allain *et al.,* 1974.[37]The absorbance was measured at 500 nm and the results were expressed as mg/dl. Results are recorded in Table (4).

Evaluation of the acute anti-inflammatory activity

The assessment of the acute anti-inflammatory effect of the ethanolic extract of the leaves of *S.taccada* was carried out according to the carrageenan-induced rat paw oedema method. [38]Twenty-four male albino Wistar rats weighing 130-150 g were divided into four groups, each of 6 animals: The first group received 1 ml of normal saline and was considered as (negative control).The second group received the reference indomethacin (20 mg/kg b.wt.) was considered as (positive control).The Third group received 50 mg/kg b.wt. of the plant extract. The fourth group received 100 mg/kg b.wt. of the plant extract. One hour later, oedema was induced by a sub-plantar injection of 0.1 ml of 1% carrageenan solution in saline in the right hind paw and 0.1 ml saline in the left hind paw. The paw volume was measured at 1, 2, 3 and 4 hrafter the induction of inflammation. The difference between initial reading (V_b) and subsequent readings (V_t) gave the change in oedema volume for the corresponding time and calculated according to the following formula: % oedema = $[(V_t - V_b) / V_b] \times 100$

Furthermore, the percentage inhibition of paw oedema in the mean of the treated groups in comparison with the control non-treated group was estimated and calculated according to the following formula:

% Inhibition = [1 - (E_t / E_c)] x 100

Where: E_c = percentage oedema of the control; E_t = percentage oedema of the treated group. The data was presented as mean ± standard error. Results were recorded in Table (5).

Table 5: Anti-inflammatory activity of the ethanolic extract of the leaves of *Scaevola taccada* (Gaertn.) Roxb.

Time (H)	1 hr			2 hr			3 hr			4 hr		
Groups	% Oedema	% Inhibtion	Potency	% Oedema	% Inhibtion	Potency	% Oedema	% Inhibtion	Potency	% Oedema	% Inhibtion	Potency
Control	31.37±0.23	0	-------	33.35±1.58	0	------	36.66±1.2	0		40.33±0.1	0	-------
Indomethacin 20mg/kg b.wt.	20.87±0.1*	33.47	100	13.56±0.36*	59.34	100	10.45±0.3*	71.49	100	8.19±0.3*	79.69	**100**
EtOHext 50 mg/kg b.wt.	27.03±0.5*	13.83	41.32	25.86±1.6*	22.45	37.83	23.54±0.2*	35.78	50.04	21.01±0.4*	47.90	**60.10**
EtOH ext.100 mg/kgb.wt.	22.48±4.4*	28.33	84.64	18.96±0.3*	43.10	72.69	13.81±1.0*	62.32	87.17	10.08 ±1.6	73.22	**91.88**

*Significantly different from the control normal inflamed group at $p < 0.05$. Potency was calculated relative to the standard drug indomethacin.

Statistical analysis

Comparisons were carried out using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparisons test. The minimal level of significance was identified at $p<0.05$.

Results

Macromorphology

Scaevola taccada (Fig.1A-B) is an evergreen, erect, dense, multi-stemmed shrub that measures about 1.5 to 3 m height; the plant flowers from June to August while the fruits appear at the middle of August till October. Main stem is hard, solid, erect and cylindrical with thick brown cork showing longitudinal fissures, transverse cracks and scattered lenticels. The branching is sympodial. The old branches are hard to break having fibrous fracture and exhibiting pale yellow interior. Young branches are glabrous, woody, cylindrical green in color, showing fine longitudinal striations (Fig.1A). Leaves are petiolate, alternately arranged, crowded at the tip of the stem. Leaf lamina is green in color, simple, obovate in shape measures from 6to 16 cm in length and from 2.5 to 6 cm in width at the widest part. Leaves have asymmetric base, obtuse apex and entire revolute margin. The venation is pinnate reticulate, the veins leave the midrib at angle 55 - 60 ºC and are running towards the margin where they anastmose, the upper surface is dark green and the lower is light green in color. The midrib is more prominent to the lower surface. Both surfaces appeared glabrous except the base showing tuft of hairs. The petiole is very short, flattened, pale green and glabrous. The branches possess a characteristic odour and slight bitter taste (Fig.1B).The inflorescence (Fig. 1B)is bore in group of three in short, axillary, cymose inflorescence. The flowers (Fig.1B); are small, zygomorphic hermaphrodite white sometimes with purple streaks, pedicellate, having short green pedicle measuring0.2-0.7cm long. The flowers have the following floral formula: $K_{(5)}$, $C_{(5)}$, A_5, G_2. Calyx (Fig.1B) is persistant, synsepalous, epigenous formed of 5 cup-shaped united green sepals. Sepals are linear in shape with entire margin and acute apex. The corolla (Fig. 1B) consists of 5 white densely hairy petals united at the base forming a tube is nearly about 3-3.5 cm long. The corolla is splits along one side giving the flowers a distinctive fan-like shape; petals are nearly obovate, pale green to the outside and white to the inside. The androecium (Fig.1B) is epigenous consist of 5 free fertile stamens; which are equal in length with basifixed anthers. The gynaecium (Fig.1B) is consisting of

synocarpous, bicarpellary bilocular, inferior ovary with axile placentation. Style is green incurved at the apex facing the fan-shaped corolla and terminated with bilobed stigma which is surrounded by a cup-shaped like structure fringed with hairs called "indusium"(Fig.1B). The flowers are odourless with no particular taste.

Micromorphology

A transverse section in the old stem (Fig.2A) exhibits a circular outline. It is formed of cork followed by a narrow secondary cortex. The endodermis is indistinct. The pericycle is formed of patches of lignified fibers occasionally interrupted by parenchyma (Fig.2A). The vascular tissue is wide representing about 1/5 of the diameter and formed of a continuous ring of open collateral vascular bundles traversed by biseriate to triseriate medullary rays. The central pith is relatively narrow, constituting about 1/4 of the diameter and containing scattered clusters and prisms of calcium oxalate. The cork (Fig.2A), it is formed of 4-6 rows of radially arranged tangentially elongated cells with thick suberized walls, appearing nearly polygonal isodiametric in surface view. The cortex (Fig. 2A), it is formed of 13-16 rows of slightly tangentially elongated thin walled parenchyma cells containing prisms of calcium oxalate and large rounded starch granules. The pericycle (Fig.2A, C), it is formed of patches of lignified fibers interrupted with thin-walled parenchyma cells. Pericyclic fibers are fusiform, long with moderately wide to narrow lumina, having straight or undulating walls and acute tapering apices.The vascular tissue (Fig. 2A), it is consists of a complete ring of open collateral vascular bundles which traversed by biseriate to triseriate medullary rays. The phloem (Fig.2A) consists of soft phloem tissue formed mainly of thin walled parenchymatous cells, sieve tubes and companion cells. Phloem parenchyma cells contain prisms of calcium oxalate. The xylem (Fig.2 A & C) is lignified and formed of radially arranged elements. Xylem vessels are mostly simple, showing lignified spiral, annular and pitted thickening. Wood fibers are fusiform with lignified walls, wide lumina and acute tapering apices. Wood parenchyma is rectangular in shape with thick pitted lignified walls. Medullary rays are biseriate to trisreiate, formed of rectangular cells having thin walls. The pith (Fig. 2A) is formed of parenchyma cells, first rows beneath the vascular bundle are rounded having pitted lignified walls while the rest of the cells are non-lignified and containing prisms of calcium oxalate.

The structure of the young stem (Fig. 2B) is almost similar to that of the old stem with the following differences: Absence of cork and presence of epidermal cells which are polygonal, slightly axially elongated to isodiametric, having straight anticlinal walls, covered with thick smooth cuticle and devoid of stomata, cortical tissue is formed of thick-walled collenchyma cells followed by parenchyma, containing prism of calcium oxalate and starch granules and the vascular tissue is much narrower while the pith is wider, and formed of rounded thin-walled parenchyma cells.

Powdered stem (Fig. 2C) is greenish brown in colour with characteristic odour and slightly bitter taste. It is characterized by the presence of the following: Fragments of brown, polygonal, suberized cork cells. Moreover, fragments of polygonal axially elongated epidermal cells having straight anticlinal walls and covered with smooth cuticle and devoid of stomata are detected. Fragments of thin walled parenchyma cells containing prisms of calcium oxalate crystals and starch granules which are simple and circular in shape are noticed. Furthermore, fragments of lignified pericyclic fibres with straight or tortuous walls showing moderately wide to narrow lumina and having acute tapering apices are present. In addition to fragments of lignified wood fibres with straight or tortuous walls having wide lumina and acute tapering apices are detected. Xylem vessels with spiral, annular and pitted lignified thickening are present.

A transverse section in the leaf (Fig.3A) shows upper and lower epidermises enclosing in between a homogenous mesophyll. The spongy tissues show

several rows of loosely arranged thin walled parenchyma cells which are loosely arranged. The midrib is more prominent to the lower surface and exhibit three crescent-shaped collateral vascular bundles accompanied by an inverted small one. The first (3-4) rows of cortical tissues are collenchymatous followed by several rows of parenchyma cells containing scattered clusters of calcium oxalate. The vascular system is surrounded with a parenchymatous pericycle. The upper epidermal cells (Fig.3 A,C) are formed of polygonal nearly isodiametric cells with straight thick anticlinal walls, covered with smooth cuticle and devoid of stomata. The lower epidermal cells are formed of polygonal isodiametric to slightly elongated smaller cells with straight anticlinal walls and covered with smooth cuticle. Stomata are of anisocytic type surrounded by three subsidiary cells with few anomocytic. Some epidermal cells show clusters and prisms of calcium oxalate. Trichomes (Fig. 3C) are present at the site of leaf base which areof non-glandular type,they are small, unicellular, unbranched and covered with smooth cuticle. The Mesophyll (Fig.3A) is homogenous undifferentiated. Spongy tissues formed of several rows of irregular shaped parenchyma cells with wide intercellular spaces and containing scattered clusters and prisms of calcium oxalate.Smaller vascular bundles of lateral vein are also present in the region of spongy tissue. Midrib (Fig.3A), consisting mainly of several rows of parenchyma cells with 3-4 rows of subepidermal collenchyma on upper surface and about 5-6 rows of collenchyma cells on the lower one. The parenchyma cell are thin walled mostly rounded in shape, some parenchyma cells containing large starch granules, scattered clusters and prism of calcium oxalate and few tannin cells are also present. The pericycle (Fig.3A), consists of thin walled parenchyma cells.The vascular tissues consists of four vascular bundles, one of them is smaller and inverted. Each one is crescent in shape and of collateral type, formed of xylem towards the upper side and phloem towards the lower one.Xylem vessels (Fig. 3A,C) are spiral and annular lignified thickening which arranged in radial rows. Tracheids (Fig.3C) are few, lignified with annular thickening. Wood fibers **(**Fig. 3C) are fusiform with lignified walls, having moderately wide to narrow lumen and acute apices. Wood parenchyma (Fig. 3C) is formed of rectangular cells having thick pitted and lignified wall. Uniseriate medullary rays (Fig. 3A, C) traverse the xylem and the phloem are formed of large rectangular radials elongated cells having thin walls and containing numerous rosettes of calcium oxalate. A transverse section in the petiole (Fig.3B) is winged in outline and formed of upper epidermis, cortical tissues showing collenchyma followed by several rows of thin walled parenchyma cells containing clusters of calcium oxalate then lower epidermis. The vascular tissues consisting of arc of several collateral vascular bundles extended to the margins of the transverse section. The epidermis (Fig.3C) consists of polygonal axially elongated cells with thick straight anticlinal walls covered with smooth cuticle. Stomata are few and of anisocytic type.

Powdered Leaf (Fig.3C) is green in color, has characteristic odor and bitter taste characterized microscopically by the presence of fragments of the upper epidermis showing polygonal isodiametric cells with thick straight anticlinal wall, covered with smooth cuticle and devoid of stomata. The lower epidermis are polygonal isodiametric to slightly elongated with thin straight anticlinal walls, covered with smooth cuticle and showing anisocytic stomata with few anomocytic. Fragments of epidermis of petiole showing polygonal axially elongated cells, few anisocytic stomata, thick straight anticlinal wall and covered with smooth cuticle are present. Fragments of non-glandular trichomes, unicellular, unbranched covered with smooth cuticle are noticed. Fragments of medullary rays are large rectangular in shape, having thin walls and containing rosettes of calcium oxalate. In addition to, fragments of lignified narrow xylem vessels with spiral, annular and

pitted thickenings. Lignified wood fibers with straight wall, moderately wide to narrow lumen and others with undulating walls and acute apex are present. Fragments of wood parenchyma, rectangular in shape having thick pitted and lignified walls. Lignifiedtracheids of annular thickening and blunt apex scattered clusters, prisms and rosette of calcium oxalate are observed. Starch granules of simple and compound type are present.

A transverse section in the flower at the upper part of the calyx representing sepals, petals, filament and style as shown (Fig.4a) sepals is planoconvex composed of outer and inner epidermises enclosing in between a homogeneous cortical parenchymatous cells, traversed by small collateral vascular bundles. The outer (lower) epidermis (Fig. 5A) consists of polygonal isodiametric cells to slightly elongated with straight anticlinal walls, covered with smooth cuticle, stomata of anomocytic type, trichomes are non-glandular and cells contain prisms and numerous rosettes of calcium oxalate. The inner (upper) epidermis (Fig.5A) consists of large polygonal isodiametric cells, with straight anticlinal walls, covered with smooth cuticle, devoid of stomata and cells contain rosettes and prisms of calcium oxalate. Trichomes (Fig.5A) are present on outer epidermis, they are non-glandular unicellular, curved and covered with smooth cuticle.

A transverse section through the corolla tube (Fig.4e,f)showed five united lobes, each lobe is nearly planoconvex, showing an outer and inner epidermises enclosing in between a homogeneous mesophyll (Fig. 4 e,f) consisting of 14-16 rows of parenchymatous cells having thin cellulosic walls containing scattered prisms and rosettes of calcium oxalate, traversed by small collateral vascular bundles (Fig.4e,f).The outer epidermisin the apical region (Fig.5G)is formed of polygonal nearly isodiametric cells, having straight to slightly wavy anticlinal walls and covered with smooth cuticle, stomata of anomoyctic type and trichomes are of non-glandular type, unicellular and covered with smooth cuticle. Cells contain prisms and numerous rosettes of calcium oxalates. In the middle region (Fig.5H), the outer epidermal cells becoming polygonal isodiametric to slightly elongated cells having straight anticlinal walls, covered with faintly striated cuticle. The outer, inner epidermal cells of the basal region (Fig.5I)are more axillay elongated with straight anticlinal walls. Trichomes are absent. The inner epidermis in the apical region (Fig.5J) is formed of polygonal slightly axially elongated cells having thick straight anticlinal wall.In the middle region (Fig.5K), epidermal cells becoming axially elongated having slightly thick straight anticlinal walls.

A transverse section in the flower at the androecium level (Fig. 4g) showed five stamens of anthers and all were enclosed the five united petals. A transverse section in the anther shows (Fig. 4j) two anther lobes attached by a connective tissue enclosing a vascular bundle. Each anther lobe has two pollen sacs containing numerous reddish brown pollen grains. The epidermis (Fig.5B)consists of polygonal isodiametric cells having thick, wavy anticlinal walls and covered with smooth cuticle. Stomata and trichomes are absent. The fibrous layer (Fig. 5B)is formed of one raw of radially elongated cells which become thickened with lignified bar-like thickenings from the side view and appear with beaded walls from the top view. The pollen grains (Fig.5B) are large spherical in shape; appearing oval from side view, having smooth exine with three germ pores and three germinal furrows. A transverse section in the filament (Fig.4i), consists of an epidermis enclosing a parenchymatous mesophyll, showing scattered rosettes of calcium oxalate, and traversed by one central collateral vascular bundle. The epidermal cells (Fig.5E) are formed of polygonal, axially elongated cells having straight anticlinal walls and covered with smooth cuticle devoid of stomata and trichomes. A transverse section in the ovary (Fig.4 l) appears bilocular and containing one large ovule attached to an axile placentation. The ovary wall consists of an epidermis enclosing parenchymatous

ground tissue, traversed by several vascular bundles (8-10) and shows prisms and numerous rosettes of calcium oxalate. The epidermal cells (Fig.6A) are formed of polygonal isodiametric cells with straight anticlinal walls and smooth cuticle and devoid of stomata. Trichomes of non-glandular type are present.

A transverse section in the style (Fig.4n) is somewhat oval in outline. It consists of an epidermis surrounding a wide parenchymatous ground tissue contains rosette of Ca ox which is traversed by 4-6 small vascular bundles. The epidermal cells (Fig.6A) of the style are polygonal axially elongated with straight anticlinal walls and covered with smooth cuticle. Stomata and trichomes are absent. The stigmatic surface (Fig.6A) shows a papillosed epidermis. The papillae are short having swollen rounded apices and covered with smooth cuticle. The indusium (Fig.6A) is a cup-shaped like structure fringed with long, numerous trichomes. It surrounds the papillosed stigma and consists of polygonal axially elongated cells with straight anticlinal walls, covered with smooth cuticle and containing rosettes of calcium oxalate. Few stomata are present. Trichomes are very abundant on indusium surface, they are non-glandular and unicellular.

A transverse section in the pedicel (Fig.4q) is round in outline. It is formed of epidermis, a cortex and vascular system which is formed of 6-8 collateral vascular bundles arranged in a circle surrounding a parenchymatous area of pith. The epidermis (Fig.6B) consists of polygonal axially elongated cells, having straight anticlinal walls and covered with smooth cuticle. Trichomes are short non- glandular unicellular covered with smooth cuticle.

The powder of the dried flower is pale yellow in color, odorless and tasteless. It is characterized microscopically by the presence of the of the outer, inner epidermis of the sepals formed of polygonal isodiametric cells to slightly elongated with straight anticlinal walls, covered with smooth cuticle, stomata of anomocytic type, trichomes are non-glandular and cells contain prisms and numerous rosettes of calcium oxalate. The inner epidermis of the sepals is devoid of stomata and cells contain rosettes and prisms of calcium oxalate. Outer epidermis of the petal are polygonal nearly isodiametric cells, having straight to slightly wavy anticlinal walls and covered with smooth cuticle, and stomata of anomoyctic type at the apical region The cells at the middle region becoming slightly elongated having straight anticlinal walls and covered with faintly striated cuticle. At the base the cells become more axially elongated. Fragments of the epidermis of the filament, style, indusium and the pedicel consist of polygonal, axially elongated cells having straight anticlinal walls and covered with smooth cuticle and no stomata. The epidermis of anther consists of polygonal isodiametric cells having thick wavy anticlinal walls and covered with smooth cuticle and devoid of stomata. Fragments of fibrous layer of anther showing polygonal elongated cells with lignified bar-like thickenings from the side view and beaded from the top view. Numerous pollen grains which are rounded or oval in shape, having smooth exine, three germ pores and three germinal furrows. The epidermis of ovary consist of polygonal isodiametric cells with straight anticlinal walls and smooth cuticle. Stomata are absent. Lignified xylem vessels having spiral and annular and pitted thickenings (from different parts of the flower) are detected (Fig.5, 6). Numerous scattered rosettes, clusters and prisms of calcium oxalate are presented. Fragments of tracheids having annular thickenings also detected. Several types of non-glandular trichomes short unicellular, having acute and blunt apices and covered with smooth cuticle as well as large unicellular, having blunt apices and covered with warty cuticle are prevailed. Microscopically measurements of the different elements of the flower of *S. taccada* are recorded in Table (1).

Genetic profiling

Unambiguous plant identification is of primary concern to guarantee quality, safety and efficacy of a drug or an extract. Our present study clearly indicated

that RAPD markers could be used effectively to authenticate the plant under investigation in the local herbal markets.

The analysis of ISSR-PCR data can thus select the use of primers HB-11and HB-13 for the selective discrimination of the Egyptian *S.taccada* cultivar from other varieties. These primers may be used as an indicator for obtaining genetic markers. RAPD analysis prevailed of nineteen bands of total different fragments. Which showing 4 bands by primer A-07 ranging from 0.3 Kbp to 0.45 Kbp , 6 bands by primer A-10 ranging from 0.1 Kbp to 0.45K bp , primer B-07 produced 3 bands ranging from 0.3K bp to 0.5 Kbp , and 5 bands are produced by primer M01 ranging from 0.25 Kbp to 0.7 Kbp (Figure 6). Moreover, a total of twenty one different fragments were obtained using ISSR analysis which showing 3 bands by primer HB-08 ranging from 0.2 to 0.3 Kbp, 3 bands by primer HB-10 ranging from 0.3 Kbp to 3.00 Kbp, 7 bands by HB-11 ranging from 0.15 to 0.5 Kbp, HB-13 shows only 4 bands ranging from 0.45 to 3 Kbp and HB-14 shows 4 bands ranging from 0.4 to 0.65 Kbp. HB-11 and A10 primers were produced the largest numbers of bands 7and 6 band with RAPD and ISSR analysis respectively. The least number of bands are produced by HB-08, HB-10, A-07primers (Figure 6). Comparison of the two PCR-dependent techniques revealed that ISSR will be more useful and informative than RAPD in identification of *S. taccada.* Our study showed that there is a large genetic distance between commercial cultivars of *Scaevola* (Purple Fanfare, Pink Perfection, and Mauve Cluster), indicating considerable genetic variation among them. The use of RAPDs in intra- and inter-specific breeding of *Scaevola* is also explored. [39] The genetic diversity of populations of **S. plumieri** within its South African range was examined using Inter Simple Sequence Repeats (ISSR). [40] We have characterized 13 microsatellite loci for **S. taccada**. These microsatellite loci will be useful for estimating population genetic structure possibly resulting from the various seed dispersal patterns of **S. taccada.**[41]

Determination of Proximate and Macronutrient Composition

Proximate analysis of the leaves was carried out to facilitate the detection of the quality and uniformity of the plant where the results showed a total ash (14.95 g%), acid insoluble ash (5.07g%), water soluble ash (5.07 g%), moisture content (9.11 g%) , total protein (12 g%) and carbohydrates (6.55 g%).The analytical standards (total ash, total proteins, total carbohydrates and moisture contents) that are reported here for the first time could serve as useful quality control criteria for conformation of identity and purity of the leaves of the plant. In addition, the leaves could be considered a good source of protein (12%) that is an important building block for muscle, hair and nails. This may explain the traditional use of *S.taccada* leaves for curing skin diseases, in consideration that protein consumption help in building and repairing body tissues. [42]

Investigation of the Lipoidal Contents

GLC analysis of the unsaponifiable matters (USM)

From (Table 2) it could be concluded that: The number of identified components in the USM of the leaves of *S. taccada* was 19 components, representing 92.4%. The hydrocarbons constitute 64.29% of the total composition and detected as a series of alkanes (ranging from C_{14}-C_{29}), where *n*-pentacosane (27.7 %) was the major identified hydrocarbon. The phytosterol content reached 25.52% of the total composition. Stigmasterol was the major identified sterol (13.17%) followed by *β*-Sitosterol (6.13%), cholesterol and campesterol were also detected (3.27, 2.98 %, respectively). The only detected triterpene was α-Amyrin (2.6%). Phytosterols are constituents present in plants that mimic cholesterol. The National Institutes of Health claim that there are over 200 different phytosterols, but the most common plant sterols are: *β*-sitosterol, campesterol, stigmasterol, Δ^5-avenasterol. FDA stated that for foods or beverages containing at least 0.4 g plant sterols, if consumed twice daily (total intake of 0.8 g/day) as a source of diet low in

saturated fat and cholesterol, may decrease the risk of coronary heart disease. The most common natural sources of phytosterols are: almonds, flaxseed, pine nut and sunflower kernels. [43] The high percentage of phytosterols (25.52 %) detected in *S. taccada* leaves may rationalize the significant anti-inflammatory and hypocholesterolemic activities evidenced by the ethanolic extract of the leaves.

GLC analysis of the fatty acid methyl esters (FAME)

Identification of the fatty acids was carried out *via* comparing the retention time of their methyl esters to those of the available reference fatty acids similarly analyzed. From results compiled in Table (3), the following could be concluded: The number of identified fatty acids in the leaves of *S. taccada* (Gaertn.) Roxb. was 16 components representing 93.15%**.**The major identified components were the saturated fatty acids which constitute 65.4% of the total composition of the saponifiable matter, while the unsaturated fatty acids represented only 27.75%. Palmitic acid was the major identified saturated fatty acid (34.60%).Linolenic (9, 12, 15-octadecatrienoic acid) was the predominant unsaturated fatty acid 12.19 % followed by linoleic (omega-6). Omega 3 fatty acids include; alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), while PUFA ω-6 include linoleic acid (LA) and arachidonic acid (AA). Several fatty esters mainly δ-amyrin fatty esters with C_{20}–C_{30} acid moieties were reported from the heartwood and bark of *Scaevola floribunda*. [10] Palmitic acid, oleic acid isolated from *S.spinescens*,while linoleic acid has been isolated from *S.taccada*. Consumption of PUFA ω-3 was found to decrease coronary heart diseases (CHD) occurrence proved by clinical trials through consumption of fish oil or purified PUFA ω-3. PUFA ω-3 not only decreases the total and LDL cholesterol, but also makes small increase in HDL cholesterol. [15]

Determination of total phenolic compounds and total flavonoidal content in the leaves

Spectrophotometric determination of the total phenolic content was carried out using the Folin-Ciocalteau method and expressed as gallic acid equivalent. The total phenolic content of the leaves was 70.59 mg GAE/g dry extract. The total flavonoid content was established by adopting the aluminium chloride colorimetric method and expressed as rutin equivalent. The total flavonoid content of the leaves was found to be 57.12 mg rutin/g dry extract. The majority of bioactive compounds reported in plant materials were phenolic compounds.[44] An optimized extraction condition for the maximum yield of phenolic compounds from *S. taccada* was previously reported to be essential for ongoing assessment of potential biological and anti-cancer activity. [44] Among the tested organic solvents, acetone (78.58 mg GAE/g) exhibited the highest extraction efficiency, followed by methanol and ethanol, which accounted for 89.2 % (70.1 mg GAE/g) and 86.5 % (68.0 mg GAE/g) respectively, of TPC extracted by acetone.[45]Our results prevailed that the TPC evidenced by the Egyptian *S. taccada* is comparable to the reported results by Voung *et al*., (2014). [45]

In- vivo biological activities

Antihyperglycemic effects

Injection of STZ in male Wistar rats resulted in a successful induction of diabetes as indicated by high fasting blood glucose level (FBG) > 230 mg/dl). Also, STZ injection caused an elevation in both cholesterol and triglycerides levels by 37.87% and 23.82% respectively. The calculated data (Table 4) revealed that administration of the ethanolic extract at a dose level of 200 mg/kg b.wt. for 2 weeks, significantly reduces fasting blood glucose (FBG) levels by 50.37% (89.59% potency) when compared to diabetic untreated rats, while the aqueous extract at dose 200 mg/kg b.wt. showed a significant reduction in FBG level by 52.56% (93.49% potency), as compared to gliclazide (10 mg/kg b.wt.)which cause a decrease in FBG by 56.22%.

Moreover, both extracts reduced the measured lipid parameters elevated by induction of diabetes. The ethanolic extract at a dose level 200 mg/kg is more potent

than the aqueous extract as it exhibits a reduction in both cholesterol and triglycerides levels by 24.71% and 18% respectively as compared to standard gliclazide (potency 72.87% and 94.69% respectively). The hypolipidemic effect of the tested extracts is crucial in prevention of diabetes complications. The antidiabetic effect of the ethanolic extract of the leaves of *S.taccada* compared to glibenclamide reported by Umadevi et al.,(2006) [17]was comparable to the establish results in this study. Results are recorded in (Table 6).

Anti-inflammatory activity

The data presented (Table 5) revealed that the ethanolic extract of the leaves at a dose level of 50 and 100 mg/kg b.wt. exhibited a significant anti-inflammatory activity with maximum activity after 4 hours with 60.10% and 91.88% potency, respectively compared to indomethacin (20 mg/kg b.wt.).By reviewing the literature and based on the phytochemical studies of *S. taccada* leaves, the significant anti-inflammatory activity of the ethanolic extract could be attributed to the presence of some phytoconstituents such as terpenes and sterols which are known to have anti-inflammatory action.[46-48]

Discussion

Precise characterization and quality assurance of starting material is an essential step to ensure reproducible quality of herbal medicine.[1] Intended for this purpose we have done a pharmacognostical and biological studies of *Scaevola taccada* (Gaertn.) Roxb. family Goodeniaceae grown in Egypt. The botanical study of different organs of *S.taccada* are in accordance for the previous reported data. [2,12] Our study showed that there is a large genetic distance between commercial cultivars of *Scaevola* (Purple Fanfare, Pink Perfection, and Mauve Cluster), indicating considerable genetic variation among them. RAPDs in intra- and inter-specific breeding of *Scaevola* is also explored. [39] The genetic diversity of populations of **S. plumieri** within its South African range was examined using Inter Simple Sequence Repeats (ISSR). [40] We have characterized 13 microsatellite loci for **S. taccada**. These microsatellite loci will be useful for estimating population genetic structure possibly resulting from the various seed dispersal patterns of **S. taccada.**[41] The nutritional constitute of the plant may explain the traditional use of *S.taccada* leaves for curing skin diseases, in consideration that protein consumption help in building and repairing body tissues. [42] The most common natural sources of phytosterols are: almonds, flaxseed, pine nut and sunflower kernels. [43] The high percentage of phytosterols (25.52 %) detected in *S. taccada* leaves may rationalize the significant anti-inflammatory and hypocholesterolemic activities evidenced by the ethanolic extract of the leaves. Several fatty esters mainly δ-amyrin fatty esters with C_{20}−C_{30} acid moieties were reported from the heartwood and bark of *Scaevola floribunda*. [10] Palmitic acid, Oleic acid isolated from *S.spinescens*, while linoleic acid has been isolated from *S.taccada*. Consumption of PUFA ω-3 was found to decrease coronary heart diseases (CHD) occurrence proved by clinical trials through consumption of fish oil or purified PUFA ω-3. PUFA ω-3 not only decreases the total and LDL cholesterol, but also makes small increase in HDL cholesterol. [15] Our results prevailed that the TPC evidenced by the Egyptian *S. taccada* is comparable to the reported results by Voung*et al*., (2014). [45] The hypolipidemic effect of the tested extracts is crucial in prevention of diabetes complications. The antidiabetic effect of the ethanolic extract of the leaves of *S.taccada* compared to glibenclamide reported by Umadevi et al.,(2006) [17] and Gheibi et al.,(2017)[46] was comparable to the establish results in this study. By reviewing the literature and based on the phytochemical studies of *S. taccada* leaves, the significant anti-inflammatory activity of the ethanolic extract could be attributed to the presence of some phytoconstituents such as terpenes and sterols which are known to have anti-inflammatory action.[47]

Conclusions

This is the first report on authentication, quality

control and biological evaluation of the plant cultivated in Egypt. Diminutive difference observed between the introduced plant and that grown in Australia, its native area (Nobbs, 2001).[12] Furthermore, the leaves evidenced potential hypoglycemic and anti-inflammatory effects. Phytochemical studies of the aqueous and alcoholic extracts of *S.taccada* leaves are recommended to identify the constituents responsible for those activities.

Conflict of interest

Authors declare no conflict of interest

Author contributions

All authors were contributed in the idea, design the study, draft the article, review the data and edit the article.

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الاستكشاف الدوائي والبيولوجي لـ جودة نبات سكافولا تاكادا(جارتن.) روكسب. المنزرع في مصر

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ملخص

معايير تحديد ومراقبة جودة نبات *سكافولا تاكادا* (جارتن.) روكسب. المنزرع في مصر كانت ضئيلة. لذلك هدفت الدراسة الحالية إلى تقييم سمات سلالة نبات *سكافولا تاكادا* المنزرع في مصر كنبات طبي تقليدي. تم عمل دراسة نباتية مفصلة للاجزاء الكاملة ومسحوق اوراق وساق وزهرة نبات *سكافولا تاكادا* لمعرفة خصائصها. تم إجراء بصمة الحمض النووي للأوراق باستخدام طريقتين تعتمدان على تفاعل البلمرة المتسلسل (PCR). تم تقدير المحتوى الكلي من الكربوهيدرات والدهون والبروتين. تعرضت الدهون للكروماتوجرافيا الغازية السائلة (GLC). تم عمل التحليل الطيفي لمحتويات البوليفينول. كشفت مقارنة نتائج الطريقتين المعتمدتين على PCR أن تكرار التسلسل البيني البسيط (ISSR) سيكون أكثر فائدة وإفادة من DNA متعدد الأشكال المضخم العشوائي (RAPD) في تحديد *سكافولا تاكادا* . أظهر التحليل التقريبي لمسحوق الأوراق المجففة المحتوى الكلي والذوبان في الماء والرماد الغير قابل للذوبان في الحمض ومحتوى الرطوبة 14.95٪ و 5.07٪ و 5.07٪ و 9.11 / وزن على التوالي. أظهر فحص القيمة الغذائية وجود نسبة عالية من البروتين بنسبة 12٪ وايضا محتوى ملحوظ من الكربوهيدرات. وقد اظهرت النتائج ان المحتوى الدهني الرئيسي يشمل n-pentacosane٪27.70 , stigmasterol ٪13.16 و α-linolenic acid 12.19 ٪. تم إجراء الأنشطة الخافضة لنسبة السكر في الدم والمضادة للالتهابات من المستخلصات الإيثانولية والمائية للأوراق. أظهر كل من المستخلصات الإيثانولية والمائية (200 مجم / كجم) تأثير ملحوظ على خفض مستوى سكر الدم على حد سواء بالمقارنة بالجليكلازيد (10 مجم / كجم) ، ساد المستخلص الإيثانولي بانخفاض متوازي في مستوى الكوليسترول والدهون الثلاثية في نموذج الستريتوزوتوسين (STZ) الناجم عن مرض السكري. علاوة على ذلك ، فإن المستخلص الإيثانولي (100 ملجم / كجم) يدل على وجود تأثير مضاد للالتهابات بنسبة 87.17 و 91.88٪ في الساعة الثالثة و الرابعة على التوالي من اعطاؤه للعلاج مقارنة بالإندوميتاسين.

الكلمات المفتاحية: الميكرومورفولوجيا، بصمة الحمض النووي، خافضات سكر الدم، مضاد للالتهابات.

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Anaerobic Power among Able-bodied Individuals versus Disabled Persons during arm cranking and Its Relationship to Hand-Grip Strength

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ABSTRACT

Aim: This study assessed Wingate test performance among disabled individuals compared to able-bodied. It also assessed the relationship between arm cranking anaerobic power and hand-grip strength.

Methods: Fifteen able-bodied men (age, 21.5 ± 1.2 y; height, 178 ± 7 cm; body mass, 77.7 ± 10.5 kg) and 11 disabled men (age, 33.6 ± 8.5 y; height, 158 ± 27 cm; body mass, 88.3 ± 22.7 kg) volunteered to take part in the study. Able-bodied participants completed two Wingate exercise tests; one leg cycling and one arm cranking. Disabled persons completed only the arm cranking Wingate exercise test. Hand-grip strength was measured for both hands for both groups.

Results: Wingate test peak (801 ± 131 W vs. 481 ± 117 W, respectively $P < 0.001$) and mean (613 ± 107 W vs. 346 ± 75 W, respectively $P < 0.001$) power output during leg cycling were significantly higher than arm cranking. Peak (481 ± 117 W vs. 410 ± 146 W, respectively $P > 0.05$) and mean (346 ± 75 W & 311 ± 111 W, respectively $P > 0.05$) power output of able-bodied participants during arm cranking Wingate test were higher than disabled persons. There was a significant relationship between peak and mean Wingate test power output during arm cranking and hand-grip strength for both hands ($P < 0.01$).

Conclusion: Wingate test performance was greater during leg cycling compared to arm cranking and in able-bodied participants compared to disabled individuals during arm cranking. There was a significant relationship between hand-grip strength and Wingate test performance for both groups. These findings indicate differences between able-bodied and disabled individuals in Wingate test performance and reveal aspects of fitness to be improved in disabled individuals.

Keywords: Wingate; Anaerobic power; Arm cranking; Disabled individuals.

1. INTRODUCTION

Anaerobic capacity is the maximal amount of ATP turnover permissible by anaerobic metabolism (by the whole organism) during a specific type of short-duration, maximal exercise (Green, 1994; Alrob, 2017). Compared to aerobic metabolism, the power produced using anaerobic metabolism is high, but can only be sustained for a short period of time (McArdle et al. 2007). Anaerobic capacity is essential in sports involving sprints, jumping and throwing such as high jump, long jump and shot put (Bajes & Al-Dujaili, 2017). Therefore, assessing anaerobic capacity in such sports is more informative than cardiovascular and endurance assessments. There are different exercise tests used to assess anaerobic capacity, such as vertical jump and long jump; however, the Wingate anaerobic exercise test might be the most used.

Before the advent of the Wingate anaerobic exercise test, a 40 s exercise test with 6 kg for men and 5 kg for women loads were applied to the flywheel of a mechanically-braked cycle ergometer was used to assess anaerobic capacity in leg cycling (McArdle et al. 2007). The Wingate exercise test was established in the 1970s (Bar-Or, 1987). The Wingate exercise test requires

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maximal (all-out) pedalling for 30 seconds against a constant braking force in leg cycling and arm cranking (Driss, & Vandewalle, 2013). In leg cycling, a frictional resistance of 7.5% body mass has been used to determine Wingate test performance; whereas in athletes 12% body mass has been used (McArdle et al. 2007). On the other hand, 4% - 5.5 kg of body mass has been used during arm cranking Wingate exercise for healthy able-bodied individuals (e.g., Ogonowska et al., 2009; Price et al., 2014). Jacobs et al. (2005) used 1%, 2% and 3% kg of body mass during arm cranking Wingate exercise test for tetraplegic persons with 5, 6, and 7 cervical spinal cord injuries. Hutzler et al. (1998) employed 3.5% of body mass during arm cranking Wingate exercise test for individuals with lower limbs impairment.

Arm exercise is an established mode of exercise testing and prescription for people who use their upper body regularly during exercise, such as rowers, swimmers and kayakers (Franklin et al., 1983; Hill et al. 2019). Arm exercise is also an adequate mode of exercise testing for those who often complete some form of work which requires significant use of the upper body, such as digging and snow shovelling (Franklin et al., 1983). It is also an appropriate mode of exercise testing and prescription for individuals who are unable to use their legs during exercise (Janssen & Hopman, 2005). For instance, individuals with paraplegia as a result of Spinal Cord Injury (SCI) or spina bifida or poliomyelitis, as well as for those with bilateral above-knee amputees (Hopman, 1994).

Arm exercise elicits lower peak values for oxygen consumption, heart rate, pulmonary ventilation, power output and a higher systolic and diastolic blood pressure during maximal exercise compared to leg cycling or treadmill running (Åstrand et al., 1965; Aminoff et al., 1997). Similar ratings of perceived exertion values were reported at the termination of arm cranking and leg cycling maximal exercise tests (Al-Rahamneh, 2010). However, at the same sub-maximal work rate or at the same percentage of VO_2peak, arms elicit higher values for oxygen uptake, heart rate, pulmonary ventilation, systolic and diastolic blood pressure, blood lactate concentration and rating of perceived exertion (Åstrand et al., 1968; Franklin et al., 1983). McArdle et al. (2007) indicated that these differences in the maximal and sub-maximal values between the two modes of exercise may be attributed to the relatively smaller muscle mass activated during upper body compared to lower body exercise.

The hand is the most dynamic and interactive part of the upper limb (Martin et al. 2015). The hands are important for sport and/or physical activity that involves catching, throwing and lifting. During arm cranking Wingate exercise test participants are requested to hold the handlebars of the arm cranking ergometer throughout the exercise test. Therefore, measuring hand-grip strength is essential in this study. Hand-grip strength is measured by the amount of the static strength that the hand generates around a dynamometer (Massy-Westropp et al. 2011). If the measurement is conducted accurately using a reliable and valid device, hand-grip strength measurement is one of the well established and approved measurements of static strength (Mathiowetz, 2002). Hand-grip strength is positively associated with body mass index (Koley et al. 2009). These authors showed that thin individuals (BMI <18.5 $kg \cdot m^{-2}$) have low hand-grip strength compared to individuals with a normal body mass index. However, since it is unclear whether hand-grip strength correlates positively with anaerobic power during upper body exercise, further research is required to address this.

It is well established that able-bodied participants have significantly higher peak values for power output and oxygen uptake (Hopman, 1994; Janssen & Hopman, 2005; Al-Rahamneh, 2010; Al-Rahamneh & Eston, 2011; Al-Rahamneh & Eston, 2012). However, it has yet to be determined whether anaerobic power is comparable among able-bodied individuals and disabled individuals and there are no studies assessing the relationship between hand-grip strength and anaerobic power during arm cranking exercise. Since anaerobic power is important for both able-bodied and

disabled persons especially in track and field events such as shot put and weight lifting. Therefore, the aim of the current study was to assess whether there was a significant difference in anaerobic power between able-bodied and disabled individuals. The second aim of the current study was to assess the relationship between anaerobic power of arm cranking exercise and hand-grip strength. It is hypothesized that able-bodied individuals would have higher anaerobic power than disabled persons. It is hypothesized that there will be a significant positive relationship between arm cranking anaerobic power and hand-grip strength.

Methods

Participants

Fifteen able-bodied men (age, 21.5 ± 1.2 y; height, 178 ± 7 cm; body mass, 77.7 ± 10.5 kg) and 11 disabled men (age, 33.6 ± 8.5 y; height, 158 ± 27 cm; body mass, 88.3 ± 22.7 kg) volunteered to take part in the study. Inclusion criteria were: (a) being physically active ≥3 times per week; (b) free of acute illnesses (e.g., flu), chronic diseases, and sport injuries; and (c) not arm-trained. Able-bodied participants were sport sciences students studying at School of Sport Sciences at the University of Jordan. Regarding the disabled participants, three had lower limbs flaccid paralysis as a result of poliomyelitis infection, four had lower limbs amputation, one had spina bifida, two had SCI with a neurological level below T6 and more than 7 years since injury and one had lower limbs paralysis as a result of cerebral palsy. Able-bodied participants were physically active (> 3 h per week), but not specifically arm-trained (e.g. swimmer) and participated in sports such as taekwondo, football and handball at both professional and recreational levels. Disabled persons were physically active and participated in sports such as wheelchair basketball, weight lifting and track and field events at both professional and recreational levels.

As arm ergometry was not a familiar mode of exercise training for both groups, a familiarization trial was conducted before the Wingate exercise test was performed. In this familiarization trial, after a proper warming up, participants were asked to exercise for 5 minutes at 30 W. Able-bodied participants visited the lab twice whereas disabled persons had one visit. On the first visit, all participants provided written informed consent and were measured for body mass (SECA, Hamburg, Germany) and height. Disabled participants were measured for height while lying down on the floor. This study was conducted with institutional ethics approval from the School of Sport Sciences at the University of Jordan. Able-bodied participants and wheelchair users performed their exercise tests in the exercise physiology laboratory at the School of Sport Sciences at the University of Jordan.

Procedures

Able-bodied participants performed two Wingate exercise tests. The first was performed during leg cycling to assess anaerobic power of lower limbs. The second was performed during arm cranking to assess anaerobic power of upper limbs. Disabled individuals performed only the arm cranking Wingate exercise tests to assess anaerobic power of upper limbs. For able-bodied participants, the two exercise tests were separated by 48 h. All participants were asked to avoid moderate and heavy intensity exercise prior to and between the exercise tests.

Exercise tests

Leg cycling Wingate exercise test

Before the exercise test commenced, subjects warmed up for 5 min with 30 W frictional selected-load being applied to the flywheel of the ergometer using a self-selected cadence. All exercise tests were performed on the same mechanically-braked cycle ergometer (Ergomedic 894 E Monark Exercise, Varberg, Sweden). The saddle height was set to allow a slight flexion (~20°) in the knee when the leg was extended. The handlebar height was set to achieve greatest comfort. Participants were informed to remain seated throughout the test and were given strong verbal encouragement to maintain an all-out effort throughout the test. Based on previous studies (e.g., Bar-Or, 1987) 7.5% of body mass was selected for the leg cycling Wingate exercise test. The weight was dropped

automatically when 120 RPM was reached.

Arm cranking Wingate exercise test

Before exercise commenced, subjects warmed up for 5 min with 20 W frictional selected-load being applied to the flywheel of the ergometer using a self-selected cadence. For able-bodied and disabled individuals, all exercise tests were performed on the same adapted Monark bike (Ergomedic 894 E Monark Exercise, Varberg, Sweden). The bike was stabilised on a table and a Biodex chair (Biodex Medical Systems, New York, USA) was used during all exercise tests for both able-bodied and disabled individuals. This chair has the advantage of moving forward, backward and upward. The bike was loaded with some bags of sand to minimize any rocking movements. For able-bodied participants, straps were used to stabilise the legs and to minimise their contribution during the arm cranking exercise tests. The midpoint of the ergometer was set at shoulder level and the distance was set to allow a slight flexion in the elbow when the arm was extended. Participants were given strong verbal encouragement to maintain an all-out effort throughout the test. Ogonowska et al. (2009) employed 5.5% and 4.5% kg of body mass in hand cycle exercise for males and females, respectively. In addition, Price et al. (2014) employed 4% of body mass during upper body Wingate exercise test for untrained-arm male participants. Therefore, 5% body mass was chosen for the arm cranking Wingate exercise test in the current study. The mass was dropped automatically when participants reached 120 RPM.

Hand-grip strength

For able-bodied participants, hand-grip strength was measured while standing with the arm fully extended and away from the body to avoid body strength contribution to the measurement. For disabled persons, hand-grip strength was measured while seated on a braked wheelchair with the arm fully extended and away from the body to avoid trunk strength contribution to the measurement. For both groups the same dynamometer was used to measure hand-grip strength (Takei Scientific Instruments, Japan). The results were recorded as kilograms taken from the digital display of the dynamometer to the nearest 0.1 kg. The digital display of the dynamometer displayed the maximum strength within a trial and the value was reset to zero before each subsequent measurement. Hand-grip strength was measured 3 times for each hand and the best of the 3 readings was recorded. More details of hand-grip strength measurement can be found in Al-Rahamneh et al. (2020).

Statistical *analysis*

Driss, & Vandewalle, (2013) indicated that peak power and mean power output were the main focus of most studies as fatigue index was the least reliable of the three Wingate test indices as it depends on aerobic performance. Therefore, the focus of this study was peak and mean anaerobic power.

A series of paired samples t-tests were used to compare absolute and relative peak and mean anaerobic power values of leg cycling to arm cranking. A series of independent sample t-test were used to compare absolute and relative peak and mean anaerobic power values of able-bodied to disabled persons. A series of independent sample t-test were used to compare hand-grip strength for both hands of able-bodied to disabled persons. Pearson moment correlation coefficient was used to assess the relationship between anaerobic power of arm cranking and leg cycling. The data were analyzed using Statistical Package for Social Sciences (SPSS) for Windows, PC software, version 16. An alpha level of 0.05 was used for all statistical tests.

Results

Anaerobic power

Absolute and relative peak and mean anaerobic power during leg cycling and arm cranking for both groups are presented in table 1.

Table 1: Absolute and relative peak and mean anaerobic power of leg cycling and arm cranking for both able-bodied and disabled individuals.

Group	Exercise mode	Peak power (W)	Peak power (W/kg)	Average power (W)	Average power (W/kg)
Able-bodied	Leg cycling	801 ± 131*	10.4 ± 1.3*	613 ± 107*	7.7 ± 0.8*
	Arm cranking	481 ± 117	6.2 ± 1.1°	346 ± 75	4.5 ± 0.7°
Disabled	Arm cranking	410 ± 146	4.6 ± 1.2	311 ± 111	3.5 ± 0.9

* Significantly higher during leg cycling than arm cranking. ° Significantly higher for able-bodied than disabled persons. Values are mean ± SD.

Paired sample t-test showed that absolute and relative peak anaerobic power values of leg cycling were significantly higher than arm cranking ($P < 0.001$). Paired sample t-test also showed that absolute mean and relative anaerobic power values during leg cycling were significantly higher than arm cranking ($P < 0.001$).

Independent samples t-test showed that there was no significant difference in absolute peak anaerobic power values between able-bodied and disabled individuals $t_{(24)} = 1.384$, $P > 0.05$). However, relative peak anaerobic power values of able-bodied were significantly higher than disabled individuals $t_{(24)} = 3.439$, $P < 0.01$. Independent sample t-test showed that there was no significant difference in absolute mean anaerobic power values between able-bodied participants and disabled individuals $t_{(24)} = 0.949$, $P > 0.05$). However, relative mean anaerobic power values of able-bodied participants were significantly higher than disabled individuals $t_{(24)} = 3.057$, $P < 0.01$.

Hand-grip strength

Hand-grip strength of right and left hands for both able-bodied and disabled individuals are presented in table 2. In addition, the relationships between peak and average anaerobic power of arm cranking exercise and hand-grip strength for both hands are presented in table 3.

Table 2: Hand-grip strength of right and left hands for both able-bodied and disabled individuals. Values are mean ± SD.

Group	Right hand-grip strength (kg)	left hand-grip strength (kg)
Able-bodied	52.7 ± 9.6	51.3 ± 9.1
Disabled	53.5 ± 15.4	48.6 ± 13.1

Independent sample t-test showed that there was no significant difference between able-bodied and disabled individuals in hand-grip strength of the right hand $t_{(24)} = 0.158$, $P > 0.05$ and left hand $t_{(24)} = 0.616$, $P > 0.05$.

Table 3: The relationship between peak and average anaerobic power and hand-grip strength for both hands. Values are Pearson moment correlation.

	Right hand-grip strength (kg)	Left hand-grip strength (kg)
Peak anaerobic power (W)	0.740*	0.772*
Mean anaerobic power (W)	0.749*	0.737*

* Significant relationship $P < 0.01$

There was a significant relationship between absolute peak anaerobic power and right hand-grip strength $r_{(24)} = 0.740$, $P < 0.01$ and left hand-grip strength $r_{(24)} = 0.772$, $P < 0.01$. There was a significant relationship between absolute mean anaerobic power and right hand-grip strength $r_{(24)} = 0.749$, $P < 0.01$ and left hand-grip strength $r_{(24)} = 0.737$, $P < 0.01$.

Discussion

The aim of the current study was to assess whether there was a significant difference between able-bodied and disabled individuals in anaerobic power values during arm cranking exercise. The second aim of the current study was to assess the relationship between hand-grip strength and anaerobic power during arm cranking exercise.

Arms cranking versus leg cycling

Relative peak and mean anaerobic power values were significantly higher in leg cycling (10.4 w/kg & 7.7 w/kg, respectively) than arm cranking (6.2 w/kg & 4.5 w/kg, respectively) $P < 0.05$. These findings are in agreement with Weber et al. (2006). These authors reported that peak and mean anaerobic power values of leg cycling (13.3 w/kg & 9.7 w/kg, respectively) were significantly higher than arm cranking (9.3 w/kg & 5.7 w/kg, respectively). In the current study the percentage of peak and mean anaerobic power of arm cranking to leg cycling were 59.9% and 56.6%, respectively. In Weber et al. (2006) study these percentages were 69.9% and 58.8%, respectively. Arm to leg anaerobic power ratio in the current study and Weber et al. (2006) study confirm Dotan & Bar-Or, (1983) findings who showed that mean anaerobic power ratio of arm to leg was 47.6% in female subjects and 56.8% in the male subjects.

This ratio of peak and mean anaerobic power of arm cranking to leg cycling are comparable to those reported for aerobic power. Franklin et al. (1983) and McArdle et al. (2007) reported that VO_2peak values of arm cranking exercise is about 60%-70% of leg cycling values. Franklin et al. (1983) also reported that heart rate max values of arm cranking exercise is about 11 beats per minute lower than leg cycling. These differences in peak and mean anaerobic power values between the two modes of exercise can be attributed to the smaller muscle mass activated during arm cranking exercise compared to leg cycling (McArdle et al. 2007). This is confirmed by Reiser et al. (2002) who showed 8% improvements in Wingate exercise test performance in standing compared to seating position. Driss & Vandewalle (2013) indicated that this increase in peak power in a standing compared to a seating position can be attributed to the additional power from the upper body which can be transferred to the lower limbs.

The peak and mean anaerobic power values during arm cranking for able-bodied subjects in the current study (6.2 w/kg & 4.5 w/kg, respectively) were lower than those values (7.96 w/kg and 5.97 w/kg, respectively) reported by Ogonowska et al. (2009). This difference might be due to the fact that Ogonowska et al. (2009) recruited 9 male swimmers for their study where this type of sport (i.e., swimming) involves arm exercise predominantly which in turn leads to higher values of anaerobic power during arm cranking exercise. Peak and mean anaerobic power of leg cycling reported in the current study (801 w & 613 W, respectively) were lower than those values (980 W & 656 W, respectively) reported by Grant et al. (2014). This difference might be attributed to the fact that leg cycling is not a familiar mode of exercise in our daily life activities like walking whereas cycling is part of daily life activities in developed countries.

Able-bodied versus disabled individuals

Relative peak and mean anaerobic power values of able-bodied subjects were significantly higher than disabled persons ($P < 0.05$). Absolute peak and mean anaerobic power values of disabled persons were 410 W and 311 W, respectively. These values are comparable to those values reported by (Hutzler et al. 1998). These authors reported that peak and mean anaerobic power among individuals with lower limbs impairment as a results of polio, spinal cord injury and amputees were 429 W and 341 W, respectively. These differences in anaerobic power are comparable to VO_2peak and peak power output values. For example, Amari & Al-Rahamneh, (in press) reported that peak power output values for able-bodied and paraplegic participants were

(114 w & 92 w, respectively). Similar findings were reported for VO_2peak (35 ml/kg/min & 29 ml/kg/min, respectively).

These differences between able-bodied and disabled individuals might be attributed to the fact that able-bodied participants are able to use their legs for stabilization and as a fulcrum from which to push (Hopman, 1994; Janssen, & Hopman, 2005). Jacobs et al. (2005) reported that peak anaerobic power was 207 w/kg, 120 w/kg and 57 w/kg among tetraplegic persons with complete spinal cord injury at the seventh, sixth and fifth cervical levels, respectively. Jacobs et al. (2005) findings confirm the fact that the greater muscle mass engaged during the exercise, the higher the work rate that can be achieved, especially for tetraplegic and paraplegic individuals.

The relationship between anaerobic power and hand-grip strength

There were no significant differences between able-bodied and paraplegic individuals in hand-grip strength values for both hands ($P > 0.05$). All able-bodied and paraplegic individuals were right-handed. The mean of hand-grip strength values reported in the current study for both groups are similar to those values (52 kg) which were reported by Baker & Davies, (2009) for fifteen healthy male participants. However, the mean of hand-grip strength values reported in the current study for both groups are much higher than those values (32 kg) which were reported by Atabek, (2014) for female handball players.

There was a significant relationship between absolute peak and mean anaerobic power and hand-grip strength for both hands ($P < 0.05$). This strong and significant relationship between hand-grip strength and anaerobic power of upper limbs is expected and not surprising as arm cranking exercise involves some type of gripping such as handlebars gripping. In addition, hand-grip strength also involves anaerobic power as participants try to squeeze the dynamometer maximally and as such they can sustain this for only a few seconds. Furthermore, the Wingate exercise test lasts for 30 s. That means in both the hand-grip strength and the Wingate exercise test assessments, the source of energy is principally anaerobic. To our knowledge, this is the first study which has assessed the relationship between hand-grip strength and anaerobic power during arm cranking exercise. However, these findings are in agreement with findings by Atabek, (2014) who reported a significant relationship between hand-grip strength and absolute peak anaerobic power (0.535, $P < 0.05$) and absolute mean anaerobic power (0.612, $P < 0.01$) during leg cycling among female handball players. In addition, Baker & Davies, (2009) observed a significant linear relationship between hand-grip strength and leg power ($r = 0.75$, $P < 0.05$).

Conclusion

Absolute and relative peak and mean anaerobic power values were significantly higher in leg cycling than arm cranking exercise ($P < 0.05$). Relative peak and mean anaerobic power values for able-bodied individuals were significantly higher than disabled individuals during arm cranking exercise ($P < 0.05$). There was a significant relationship between absolute peak and mean anaerobic power of arm cranking exercise and hand-grip strength for both hands ($P < 0.05$). Anaerobic power of upper limbs should be a focus of coaches and personal trainers for able-bodied and disabled individuals especially in short-duration events such as weight lifting.

Conflict of interest

The author declares that there is no conflict of interest. .

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القدرة اللاهوائية على الدراجة الثابتة للذراعين لدى الافراد الاصحاء مقارنة بالافراد ذوي الاعاقة وعلاقتها بقوة القبضة

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ملخص

الهدف: هدفت هذه الدراسة التعرف الى مستوى القدرة اللاهوائية على الدراجة الثابتة للذراعين للافراد الاصحاء ومقارنتها بقيم الافراد المعاقين. وهدفت هذه الدراسة كذلك الى معرفة قوة العلاقة بين القدرة اللاهوائية على الدراجة الثابتة للذراعين وقوة القبضة.

الطريقة والاجراءات: خمسة عشر شخص من الاصحاء (21.5 ± 1.2 سنة؛ 178 ± 7 سم؛ 77.7 ± 10.5 كغم) و 11 شخص من ذوي الاعاقة الحركية (33.6 ± 8.5 سنة؛ 158 ± 27 سم؛ 88.3 ± 22.7 كغم) تطوعوا للمشاركة في الدراسة. الافراد الاصحاء قاموا بتطبيق اختبارين ونجيت الاول على الدراجة الثابتة للقدمين والثاني على الدراجة الثابتة للذراعين. اما الافراد ذوي الاعاقة الحركية فقاموا بتطبيق اختبار ونجيت على الدراجة الثابتة للذراعين فقط. وتم قياس قوة القبضة لليد اليمين واليسار من وضع الوقوف والذراع مفرودة الى جانب الجسم للمجموعتين.

النتائج: اظهرت النتائج ان القدرة اللاهوائية المطلقة كانت اعلى للقدمين (801 ± 131 وات) مقارنة بالذراعين (481 ± 117 وات). واظهرت النتائج ان متوسط القدرة اللاهوائية كانت اعلى للقدمين (613 ± 107 وات) مقارنة بالذراعين (346 ± 75 وات). واظهرت النتائج ان القدرة اللاهوائية المطلقة كانت اعلى للافراد الاصحاء (481 ± 117 وات) مقارنة بالافراد ذوي الاعاقة (410 ± 146 وات). واظهرت النتائج ان القدرة اللاهوائية المطلقة كانت اعلى للافراد الاصحاء (346 ± 75 وات) مقارنة بالافراد ذوي الاعاقة (311 ± 111 وات). واخيرا اظهرت النتائج وجود علاقة دالة احصائيا بين القدرة اللاهوائية على الدراجة الثابتة للذراعين وقوة القبضة لليد اليمنى واليسرى.

الاستنتاجات: القدرة اللاهوائية للقدمين اعلى من الذراعين. القدرة اللاهوائية للافراد الاصحاء اعلى من الافراد ذوي الاعاقة. وجود علاقة دالة احصائيا بين القدرة اللاهوائية على الدراجة الثابتة للذراعين وقوة القبضة. هذه الفروق بين الافراد الاصحاء وذوي الاعاقة تؤكد الفروق الموجدة بين الفئتين وبناءا عليه يجب العمل عرى رفع مستوى اللياقة البدنية للافراد ذوي الاعاقة الحركية.

الكلمات الدالة: اختبار ونجيت؛ القدرة اللاهوائية؛ الدراجة الثابتة للذراعين؛ الافراد ذوي الاعاقة الحركية.

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Commonly used herbal remedies for the treatment of Primary Dysmenorrhea and Heavy Menstrual Bleeding by herbalists in Amman, Jordan: A cross-sectional survey

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ABSTRACT

Background: Traditional herbal remedies are commonly prescribed by the local herbalists for female customers complaining of varied health problems. **Objectives:** The main goal of this study is to assess the perception of herbalists on the use of herbal remedies for the treatment of feminine health disorders; and to prepare two lists of the most commonly used herbal remedies for the treatments of Primary Dysmenorrhea (PD) and Heavy Menstrual Bleeding (HMB). **Method:** A questionnaire to investigate the beliefs, knowledge and behaviors of a random sample of local herbalists (n=53), on herbal remedies used for the treatment of feminine health disorders was used. Remedies used for the treatments of PD and HMB were reviewed using previously published evidence-based pharmacological studies. **Results:** Most of the herbalists (68.0%) do frequently get requests for herbal remedies to treat different feminine health disorders. Around two third of the herbalist (66.6%) showed to depend on their work experience as their main source of information on herbal medicine. Of the used herbal remedies, only *Zingiber officinale* was found clinically effective for the treatment of HMB. While herbalists recommended for 16 herbal species for the treatment of PD, *Cinnamomum ceylanicum, Foeniculum vulgare, Trigonella foenum-graecum*, and *Zingiber officinale* proved their benefits in reducing menstrual cycle complications. These treatments were associated with different mechanisms of actions**. Conclusion:** This study revealed that herbal remedies are commonly used for the treatments of feminine health disorders among the herbalists. Also, these findings shed the light on different herbal treatments that are associated with evidence-based pharmacological studies, for their efficiency and safety in the treatment of PD and HMB symptoms. As such, these treatments would be used as alternatives to the NSAIDs and other hormonal treatments, with not only lower side effects, but also with higher patients' acceptance.

Keywords: Jordan, traditional herbal remedies, heavy menstrual bleeding, primary dysmenorrhea.

1. INTRODUCTION

Primary dysmenorrhea (PD) and heavy menstrual bleeding (HMB) are very common complaints among women in their reproductive life. [1, 2] Both PD and HMB can interfere with women's life adversely affecting its quality socially and professionally.[3]

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PD is defined as a lower abdominal pain with menstruation that is usually cramping in nature, typically begins only after ovulatory cycles are established.[4] It is one of the most common complaints by young women attending gynecologic clinics, affects more than 50% of menstruating women.[5] HMB is defined as excessive menstrual blood loss. [6] It was found the fourth most common reason for secondary gynecological referrals in England and Wales, with a prevalence of 20-30% among women.[7] Treatment is tailored according to the cause in addition to the patient's need. However, medical

treatments are still the first line of therapy. The most commonly implemented medical therapies are either hormonal (combined oral contraceptive pills and progestin's) or non-hormonal (NSAIDs).[8]

At the national level, a study performed in 2015[9], showed that the prevalence of primary dysmenorrhea in female university students aged between 19 and 24 years was 55.8%. In another similar study conducted in Jordan [10], revealed that the majority of these patients were using analgesics (90.1%); including Ibuprofen (42%), Paracetamol (34%), Diclofenac salts (10%) and Prifinium bromide (5%).

NSAIDs have side effects, especially in long-term administration. The most common side effect is gastrointestinal tract irritation. Kidneys are also involved. On the other hand, most of the young women have no tendency to use hormones to reduce pain or bleeding.[9] Therefore, from the women's perspective, herbal medicines with fewer side effects are preferred over pain killers. Commonly, those young females may be unaware of the safe and efficient use of these treatments, Issa and Bashiti [11] has previously shown that most of the patients living in Jordan, especially the females, were depending on the herbalists as their main source of information, when using herbal treatments. Therefore, it is extremely important to medically address these complaints according to the patient's needs and demands, in order to ensure the safe and efficient use of these herbal treatments.

Many of the traditional medicinal systems are still depending on the use of different plant species for the treatment of PD and HMB, which belong to different families and Genes. The majority of these herbs were acting as uterine spasmolytics, or as spasmogenics, used in different forms, including powder, extracts or as herbal teas. [12] Of the studies herbal treatments are *Blue cohosh*, *Equisetum arvense*, *Hydrastis canadensis*, *Rubus idaeus*, *Vinca major*, *Achilla millefolium*, *Zingiber officinale*, *Cinnamomum ceylanicum, Vitex-agnus castus, Foeniculum vulgare, Matricaria aurea, Trigonella foenum-graecum, Echinophora platyloba, Valeriana officinalis, Zataria multiflora, Stachys lvandulifolia, Anethum graveolens, Apium graveolens, Crocus sativus, Pimpinella anisume* and Pine. These plants were found to be effective in reducing menstrual cycle pain and complications. [13, 14]

Up to our knowledge, the ethnobotanical practices, beliefs and knowledge of herbalists, on using herbal remedies for the treatment of feminine health disorders were not explored yet. Of special interest are the herbal remedies commonly used for the treatment of PD and HMB.

Study settings and design

Research tool (questionnaire)

This cross-sectional study was conducted in Amman; Jordan. The study was conducted over two months, between January and February 2019.

A face-to-face interview to complete the questionnaire was developed by the principal researchers and reviewed by three research experts. The questionnaire was then piloted with 10 medical students and academics to test for clarity and logical flow of the questions.

The developed questionnaire was designed to collect herbalists demographic information including age, gender, level of education, practicing years (work experience), and their work location (to identify the area's socioeconomic status).

The second part of the questionnaire included six closed (using 5 Likert scales) investigating their knowledge, behaviors, and beliefs in regard to herbal remedies used for the treatment of feminine health disorders.

Two open-ended questions on the most commonly used herbs for the treatment of patients with PD or HMB symptoms were also reported. The symptoms for the PD and HMB, and the differences between them were verbally explained for the herbalists' prior to answering these questions.

Study sample

According to Oran and Al-Eisawi [15], the quality of the herbal shops were determined to be either as a specialized herbal shops that are selling herbal products, incense, and spices, or as a non-specialized herbal shops, who are selling herbs in addition to other grocery products. A random sample of herbalist shops distributed among the East and the West of Amman, managed by a professional full-time herbalists (usually with 1 to 2 assistants) was identified by the research team. As there were no official statistical data available on the numbers of the registered herbalists' shops in the city of Amman, a convenient sample of 53 herbalists was included in the study.

Herbalists were interviewed at their herbal shops, and a face to face questionnaire was completed following verbal consent, without time limit and according to ISE code of ethics (www.ethnobiology.net/ethics.php). The Arabic language was used for the questionnaire and the interview. Participants were informed that all information provided was completely confidential and the results would only be presented anonymously.

Data collection regarding the used herbal remedies was comprised of the local Arabic common names of the plants; and the indication for their use. The Arabic names of these plant species were used to search for their botanical names, utilizing the previously published studies [15-19]; that investigated the available plant species in the Jordanian market (regardless of their sources), and as reported by other researchers in the Royal Society for the Conservation of Nature.

Herbal remedies composed of mixed herbs were excluded from this study, as the exact compositions of these mixtures were unknown.

Literature Review

A literature review was conducted for the plant species that have been mentioned by the interviewed herbalists using their botanical names. The available information about different in vivo, in vitro and clinical studies of these species were collected from various electronic sources like PubMed, SciFinder, Elsevier, Springer, Scopus, Science Direct, Google Scholar and Web of Science, apart from these locally available books and peer-reviewed journal were also used to collect information.

The findings of this review will be used to evaluate the benefits of the used herbal remedies for the treatment of patients with PD and HMB. Also, it can be used to increase the awareness of the users on the importance of using only the scientifically evaluated evidences regarding the efficiency and safety of herbal remedies, by launching campaigns which would discuss these findings in order to prevent and protect female users of herbal therapy.

Data analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS version 16, Chicago, IL, US). Statistical tests including one-way analysis of variance were conducted to determine the associations between socio-demographic variables and knowledge/ perception of study participant regarding herbal treatments. The descriptive analysis was carried out using percentage for qualitative variables. The chi-square test was used to calculate P-values for categorical variables. P -values of less than 0.05 were considered significant.

Results

Socio-demographic characteristics of the herbalists

A total of 53 herbalists participated in the study, with no female herbalists were found. The majority (54.8%) of the herbalists were allocated in East of Amman (lower socioeconomic areas). The mean age of the herbalists was 42.86 years, with the highest frequency (37.8%) being in the age range of 40-49 years. Data showed that the highest proportion (43.4%) of the herbalist's had more than 10 years of experience. As for education, 43.4% were holding a high school degree only, with no education qualification degrees.

Table 1. Demographic characteristics of herbalists involved in the study

Independent variable	Frequency (n=53)	Percent (%)
Age		
18 – 29	6	11.3 %
30 – 39	12	22.6 %
40 – 49	20	37.8 %
50 – 59	12	22.6 %
60+	3	5.7 %
Gender		
Male	53	100%
level of education		
Primary school	11	20.8 %
Secondary school	8	15.1 %
High school	23	43.4 %
Diploma	5	9.4 %
Bachelor's degree	6	11.3 %
Work experience		
Less than 5 years	11	20.8 %
5 – 10 years	19	35.8 %
More than 10 years	23	43.4 %
Residential area		
West of Amman	24	45.2 %
East of Amman	29	54.8 %

Herbalist's perceptions on the use of herbal medicine

Herbalists' knowledge, beliefs, behavior and practices toward herbal remedies used for the treatment of feminine health disorders are shown in Table 2. This study showed that years of experience made no significant effect on the perception and level of recommendations made by the herbalists while dispensing herbal remedies for female patients with feminine health disorder. On the other hand, regardless of the duration of their work experience, most of the herbalist (66.6%) showed to depend on their work experience as their main source of information on herbal medicine, followed by (25.0%) of them used the internet as a source of information.

Many herbalists (60.4%) do frequently (always/usually) get requests for herbal remedies to treat different health conditions by female patients related to their feminine health, as the majority (68.0%) of these patients reached desperation stages with their conventional therapy used. Consequently, herbalist would also make recommendations to customers who are seeking alternative treatments for feminine disorders on always/usually basis (64.1%). As expected, older herbalists with a longer work experience reported making more often (69.4%) recommendations on dispensed

herbal medicine compared to the younger herbalists with shorter work experience.

As such, more than half of the herbalists (60.3%) believe that the use of herbal remedies by females with health disorders could help in the treatment of their symptoms, or in reducing the dose of their conventional medicines. And therefore, about half of the herbalists (52.9%) declare their preferences to use herbal treatment over conventional medicine.

Table 2: Cross tabulation of herbalist's perceptions toward herbal remedies used for treatment of feminine health disorders based on the length of their work experience (n = 53)

	Frequencies (%)				
	<5 (n = 11)	5-10 (n= 19)	>10 (n = 23)	Total (n = 53)	p- value
Herbalists distribution between the two areas of study (East Amman: West Amman)					
	54.5:45.5	57.8:42.2	52.1:47.9	54.8:45.2	0.636
Do you get requests for herbal remedies to treat different health conditions by female patients related to their feminine health?					
Always	18.2%	21%	26.1%	22.6%	0.458
Usually	36.4%	36.8%	39.1%	37.8%	
Often	27.2%	21%	21.7%	22.6%	
Seldom	9.1%	15.8%	28.7%	11.3%	
Rarely	9.1%	5.3%	4.3%	5.7%	
Do you make recommendations or dispensed herbal medicine from your shop to your customers seeking alternative treatments for their feminine health?					
Always	18.2%	15.8%	30.4%	22.6%	0.333
Usually	45.5%	42.1%	39.1%	41.5%	
Often	18.2%	26.3%	17.4%	20.8%	
Seldom	9.0%	10.5%	13.0%	11.3%	
Rarely	9.0%	5.3%	0%	3.8%	
Do you get inquiries from these patients on the use of specific herbal remedies due to reaching desperation stages with their conventional drug therapy?					
Always	27.3%	21.1%	17.4%	20.8%	0.468
Usually	45.5%	42.1%	47.8%	47.2%	
Often	9.1%	21.1%	21.7%	18.9%	
Seldom	9.1%	5.3%	8.7%	7.5%	
Rarely	0%	10.5%	4.3%	5.6%	

What is the source of your information? If the previous questions were "always" or "Usually"					
Book	0%	0%	6.7%	2.8%	0.839
TV and radio	0%	0%	0%	0%	
Supplier agency	11.1%	0%	6.7%	5.6%	
Internet	22.2%	33.4%	20.0%	25%	
Social media	0%	0%	0%	0%	
Experience	66.6%	66.6%	66.6%	66.6%	
Do you believe that the use of herbal remedies by these patients could help in the treatment or in reducing the dose of their conventional medicines?					
Always	18.2%	26.3%	21.7%	22.6%	0.765
Usually	45.5%	31.6%	39.1%	37.7%	
Often	18.2%	21.1%	26.1%	22.6%	
Seldom	9.0%	10.5%	8.7%	9.4%	
Rarely	9.0%	10.5%	4.3%	7.5%	
Do you prefer the use of herbal treatment over conventional medicine?					
Always	27.3%	21.1%	17.4%	20.8%	0.727
Usually	36.4%	26.3%	34.8%	32.1%	
Often	18.2%	21.1%	26.1%	22.6%	
Seldom	9.0%	15.8%	13.0%	13.2%	
Rarely	9.0%	15.8%	8.7%	11.3%	

Science- based evaluation of herbal remedies used for treatment of HMB and PD

Table 3 and Table 4 summarize the available information about different in vivo, in vitro and clinical studies for the commonly used herbal remedies, investigating their effects on HMB and PD treatment, respectively.

In this study, herbalists recommended for 14 different herbal remedies to treat HMB (Table 3).The most prescribed herbs were *C. ceylanicum* (45.2%), *Arum palaestinum* (32%), and *Anastatica hierochuntica* (24.5%). Unfortunately, none of the top three prescribed herbs, was found to have any previously published evidence-based pharmacological studies,to proof the claimed benefits upon using these herbs for the treatment of HMP symptoms, or reducing the amount of menstrual cycle bleeding.

Although, only a small percentage of herbalists (11.3%) prescribed *Z. officinale* for the treatment of HMP, a randomized placebo-controlled clinical trial [20] showed a significant decrease in the amount of menstrual cycle bleeding among the patients who used *Z. officinale* over placebo.

While herbalists recommended for 16 different herbal remedies for the treatment of PD (Table 4). The most frequently prescribed herb was *C. ceylanicum* (28.3%). Recently, both in vivo and clinical studies showed a

significant benefit for this herb in reducing the intensity of PD. They also revealed to prescribe other herbs including; *F. vulgare, T. foenum-graecum,* and *Z. officinale* as an effective treatment for PD. Several clinical studies proved the benefits for these herbs as effective and safe treatments for reducing menstrual cycle pain and therefore, it can be used in the treatment of PD.

Of the plant species that were not specifically studied for their efficiency in decreasing the symptoms of PD, but still poses different mechanisms that may be potentially used in decreasing the symptoms of PD, including the extract of *A. hierochuntica* that was found to increase level of estrogen with antinociceptive action and anti-inflammatory effects. In addition, *P. dactylifera* extract was showed to increase estrogen and progesterone hormones.

Although the bark extract of *Pinus pinea* has not been previously evaluated for its use in the treatment of PD, extracts of other species of the genus *Pinus* were used for the preparation of the oil extract commercially known as Pycnogenol®. This extract has showed a potential analgesic effect on menstrual pain.[21] and benefits persist even after discontinuation.[22]

Unfortunately, *A. palaestinum* and *Origanum syriacum* were of the commonly prescribed herbs for the treatment of PD, with not any available evidence-based pharmacological studies in the literature to support the beneficial use of these herbs in reducing PD complications.

In regard to the source of these plants, they were mainly native to Jordan, except of *C. ceylanicum*, *Z. officinale, A. racemosa* and *P. pinea* were imported from different sources.

Table 3.Evidence-based pharmacological studies for the use of herbal remedies for the treatment of patients with HMB, based on different in vivo, invitro and clinical studies

Herbal products (Arabic local name)	Family	Source	Herbalists (%) (n=53)	Scientific-based evidences
Cinnamomum ceylanicum (Qirfah)	Lauraceae	Introduced from Srilanka	45.2%	No documents
Arum palaestinum (Luf)	Araceae	Native	32%	No documents
Anastatica hierochuntica (Kafmaryam)	Brassicaceae	Native	24.5%	No documents
Zingiber officinale (Zanjabil)	Zingiberaceae	Introduced from India	11.3%	Randomized, placebo-controlled, clinical trial indicates an effective complementary treatment for heavy menstrual bleeding.[20]

Herbal products (Arabic local name)	Family	Source	Herbalists (%) (n=53)	Scientific-based evidences
				Placebo-controlled, clinical trial, has shown A significant decrease in the amount of hemorrhage premenstrual cycle.[23]
				Controlled, clinical trial found ginger may be considered as an effective therapeutic option for heavy menstrual bleeding.[24]
Actaea racemosa (Black cohosh)	Ranunculaceae	Introduced from America	11.3%	No documents
Thymus capitatus (ZaterFaresy)	Lamiaceae	Native	9.4%	No documents
Trigonella foenum-graecum (Hulbah)	Leguminosae	Native	7.5%	No documents
Coriandrumsativum (Kwzbarah)	Umbelliferae	Native	7.5%	No documents
Matricariaaurea (Babonej)	Compositae	Native	5.7%	No documents
Salvia triloba (Meirameieh)	Labiatae	Native	5.7%	No documents
Starch (Nasha)*			5.7%	No documents
Ficus carica (Al-Tīn)	Moraceae	Native	5.7%	No documents
Pinus pinea (Sanubir)	Pinaceae.	Un defined	3.8%	No documents
Phoenix dactylifera (Tamur)	Palmaceae	Native	1.9%	No documents

*starch was reported as natural inert polysaccharide obtained from plant origin and available at the herbalists' shops

Table 4.Evidence-based pharmacological studies for the use of herbal remedies for the treatment of patients with PD, based on different in vivo, invitro and clinical studies

Herbal products	Family	Source	Herbalists (%) (n=53)	Scientific-based evidences
Cinnamomum ceylanicum (Qirfah)	Lauraceae	Introduced from Srilanka	28.3%	Clinical trial found Cinnamon can reduce the intensity of primary dysmenorrhea.[25]
				An in vivo study on isolated rats had shown significantly decreased the spontaneous uterine contractions.[26]
Arum palaestinum (Luf)	Araceae	Native	22.6%	No documents
Origanum syriacum (Za'tar)	Labiatae	Native	17%	No documents
Anastatica hierochuntica (Kafmaryam)	Brassicaceae	Native	24.5%	*A. hierochuntica* aqueous extract was tested in rabbit females; finding suggests that extract may increase level of estrogen. [27] Evaluation of the antinociceptive effects of the essential oil from aerial parts of *A.hierochuntica* in experimental models, found a partial blockage of the antinociceptive action by naloxone, suggests that its participation of an opioid mechanism.[28]

Herbal products	Family	Source	Herbalists (%) (n=53)	Scientific-based evidences
				In a study investigated the antinociceptive and anti-inflammatory effects of the aqueous extract and the chloroform fraction of *A. hierochuntica* plant in Swiss albino mice and Wistar rats, both extracts have significantly inhibited edema formation, demonstrated that *A. hierochuntica* has potential central and peripheral antinociceptive effects as well as anti-inflammatory activity. (29)
Foeniculum vulgare **(Shumar)**	Umbelliferae	Native	15%	Clinical trial indicates that *F. vulgare* can be used to relieve dysmenorrheal signs and menstrual duration.(30)
				Clinical trial suggested *F. vulgare* as a safe and efficacious plant for reducing menstrual cycle pain and it can be used in the treatment of dysmenorrheal.(31)
				A study found *F. ulgare* has useful effects such as anti-inflammatory, antipyretic, and analgesic.(32)

Herbal products	Family	Source	Herbalists (%) (n=53)	Scientific-based evidences
Matricaria aurea (Babonej)	Compositae	Native	13.2%	No documents
Actaea racemosa (Black cohosh)	Ranunculaceae	Introduced from America	7.5%	A randomized controlled trial shows lack of support for their use for dysmenorrhea.[33]
Thymus capitatus (ZaterFaresy)	Lamiaceae	Native	5.7%	No documents
Trigonella foenum-graecum (Hulbah)	Leguminosae	Native	5.7%	A clinical study found *T. foenum-graecum* has great importance in treating dysmenorrhea.[34]
				A prospective, open-labeled, randomized, standard-controlled study suggested *T. foenum-graecum* as efficacious, safe, cost effective, and well tolerated treatment for dysmenorrhea.[35]
				Controlled Trials study indicates very limited evidence of effectiveness as a treatment for dysmenorrhea.[36]
Sesamum indicum (Simsim)	Pedaliaceae	Introduced from India	3.8%	No documents
Starch (Nasha)			3.8%	No documents
Petroselinum sativum (baqdunis)	Umbelliferae	Un defined	3.8%	No documents
Coriandrum sativum (Kwzbarah)	Umbelliferae	Native	1.9%	No documents
Pinus pinea		Un defined	1.9%	Randomized controlled

Herbal products	Family	Source	Herbalists (%) (n=53)	Scientific-based evidences
(Sanubir)				clinical trial showed that *Pycnogenol* has a potential analgesic effect on menstrual pain.[21]
				A controlled clinical study found the analgesic-sparing effect increases with duration of supplementation and benefits persist even after discontinuation.[22]
Zingiber officinale (zanjabil)	Zingiberaceae	Introduced from India	1.9%	Crossover clinical trial study found *Z. officinale* effective in relieving pain in girls with primary dysmenorrheal.[37]
				Randomized controlled clinical trials provide suggestive evidence for the effectiveness of ginger powder during the first 3-4 days of menstrual cycle for primary dysmenorrhea.[38]
				A clinical study shows a limited evidence of effectiveness.[36]
Phoenix dactylifera (Tamur)	Palmaceae	Native	1.9%	In a study performed on female vistar rats, *P. dactylifera* extract was given in peritoneum for 14 days. Results show that increasing the amount of estrogen and progesterone hormones.[39]

Discussion

In agreement with previous studies performed by Abdelhalim *et al.* [40] and Issa & Bashiti [11], this study showed that herbal medicine continued to be commonly used by many herbalists in Jordan. The herbalists who participated in this research appear to usually prescribe varied species of herbs for their customers complaining of different women's health complications, including treatments for patients with symptoms of HMB and PD.

On the other hand, most of the herbalists revealed that their source of information on the use of herbal treatments was their work experience. For that reason, health care providers including medical doctors (MD), nurses and pharmacists are requested to play an essential role in increasing patient's awareness on the safe and efficient use of herbal remedies.

Of the commonly used herbal treatments that had previously proved to be effective in the treatment of PD and HMP was *Z. officinale*, which had clinically demonstrated a significant decrease in the symptoms related to these disorders compare to placebo[36-38] , with similar effect to that for ibuprofen in relieving menstrual pain.[41, 42] Although *C. ceylanicum* was highly recommended in the treatment of both disorders, it failed to prove its efficiency in the treatment of HMB, due to the lack of studies investigating this use. Nevertheless; clinically it demonstrated its efficacy in decreasing the symptoms of PD [25, 26], with no specific mechanism of action was determined yet [43].

Clinical trials found that *F. vulgare* and,*T. foenum-graecum* as efficacious, safe, and cost effective treatments for PD. In addition, *A. hierochuntica and P. dactylifera* extracts were showed to increase levels of estrogen and/or progesterone hormones, with antinociceptive action, analgesic and anti-inflammatory effects, in invivo studies. Therefore, these treatments may be considered as potential treatments for PD, if farther clinical studies were conducted, in order to determine the safe and efficient does.

While Pycnogenol®, is a commercial product prepared from extract of different *Pinus species* composed of oligomers of five to seven flavan-3-ol units [44], has shown a potential analgesic effect on menstrual pain[21].Wither the extract prepared from the *P. pinea* that is used for the treatment of PD and HMP has similar effect to Pycnogenol; could not be evaluated in this study.

In this study, herbal treatments used for PD and HMB complications appear to have different mechanism of action. Mainly linked to reducing menstrual blood loss, pain intestacy and duration of the period, as well as the inhibition of uterine contractions. Hormonal effects by increasing the levels of estrogen and/ or progesterone hormones were also reported. In addition, bonding to opiate receptors, with analgesic, anti-fever, and anti-inflammatory effects were also potential mechanisms.

Therefore, varied herbal women's treatment options can be available, if an appropriate clinical trials of potentially useful herbal medicines were further investigated.[45] As such, elucidation of the efficacy, potential side effects or herb-drug interactions, dosage and pharmaco-therapeutic actions of these treatments are necessary.

Significant statement

Results from this study emphasize on the need for increasing the knowledge and improving the practice of herbalists when it comes to dispensing herbal remedies for the treatment of different feminine health disorders; especially PD and HMB conditions, to ensure the safe and efficient use of these treatments. What's more important is to provide female patients with sufficient information on the use of herbal treatments, in order to protect them from the possible unwanted side effects or treatments complications.

Conclusion

Herbal treatments have been widely used among the herbalists in Amman-Jordan, for the treatment of different feminine health disorders, including PD and HMB symptoms. Although a number of herbal treatments

mentioned by these herbalists had previously been studied for their efficiency, the majority of these herbs have not been studied yet for these indications. Therefore, further scientific evaluations are necessarily, as these drugs stand as potential effective and safe treatments for PD and HMB symptoms.

Limitations

It was difficult for external validity to justify the generalization from this small sample size. Therefore, an extended study with a larger sample size is required.

Due to patient's privacy issues, we could not interview those who used herbal remedies for the treatment of PD or HMB symptoms. Therefore, further survey study on female patients, who used these treatments previously, is required in order to investigate the benefits of these remedies on the treatment of PD or HMB symptoms.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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تصميم وتوصيف الأغشية عبر العلاجات العشبية شائعة الاستخدام لعلاج عسر الطمث ونزيف الحيض من قبل المعالجين بالأعشاب في عمان ، الأردن: دراسة مقطعية لأمليديبين بيزيلات لتعزيز الفعالية العلاجية

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ملخص

المقدمة: عادة ما يصف المعالجون بالأعشاب المحليون العلاجات العشبية التقليدية للزبائن اللاتي يشتكين من مشاكل صحية متنوعة. **الأهداف:** الهدف الرئيسي من هذه الدراسة هو تقييم تصور المعالجين بالأعشاب حول استخدام العلاجات العشبية لعلاج الاضطرابات الصحية النسائية. وإعداد قائمتين من العلاجات العشبية الأكثر استخدامًا لعلاج عسر الطمث (PD) ونزيف الحيض (HMB). المنهجية: تم استخدام استبيان للتحقيق في معتقدات ومعرفة وسلوكيات عينة عشوائية من المعالجين بالأعشاب المحليين (ن = 53) حول العلاجات العشبية المستخدمة في علاج الاضطرابات الصحية الأنثوية. تمت مراجعة العلاجات المستخدمة في علاجات PDو HMBباستخدام الدراسات الدوائية القائمة على الأدلة المنشورة مسبقًا. النتائج: معظم المعالجين بالأعشاب (68.0٪) يتلقون طلبات في كثير من الأحيان للعلاجات العشبية لعلاج الاضطرابات الصحية النسائية المختلفة. أظهر حوالي ثلثي المعالجين بالأعشاب (66.6٪) أنهم يعتمدون على خبرتهم العملية كمصدر رئيسي للمعلومات عن طب الأعشاب. من العلاجات العشبية المستخدمة ، تم العثور فقط على Zingiber officinaleالزنجبيل فعالًا سريريًا لعلاج HMBفي حين أوصى المعالجون بالأعشاب بـ 16 نوعًا من الأعشاب لعلاج PD، أثبتت Cinnamomum ceylanicumالقرفة و Foeniculum vulgare شومر و Trigonella foenum-graecumالحلبة و Zingiber officinale الزنجبيل فوائدها في الحد من مضاعفات الدورة الشهرية. ارتبطت هذه العلاجات بآليات مختلفة للعمل. **الخلاصة:** كشفت هذه الدراسة أن العلاجات العشبية تستخدم بشكل شائع لعلاج اضطرابات صحة المرأة بين المعالجين بالأعشاب. أيضًا ، تلقي هذه النتائج الضوء على العلاجات العشبية المختلفة المرتبطة بالدراسات الدوائية القائمة على الأدلة ، من أجل فعاليتها وسلامتها في علاج أعراض PDو HMB. على هذا النحو، يمكن استخدام هذه العلاجات كبدائل لمضادات الالتهاب غير الستيروئيدية والعلاجات الهرمونية الأخرى ، ليس فقط مع آثار جانبية أقل ، ولكن أيضًا مع قبول من المرضى بشكل أكبر.

الكلمات الدالة: الأردن ، العلاجات العشبية التقليدية ، نزيف الحيض ، عسر الطمث.

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Determination of iron in liposomal dosage forms by flame atomic absorption spectrometry after an acidic digestion

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ABSTRACT

Improving compliance to oral iron is one of the essential goals. Iron Encapsulation into liposomes as nano-particles has provided newer opportunities for ameliorating tolerance with iron oral therapy. Several analytical techniques have been used for quantitative determination of iron. In this study, the liposomal iron has been determined quantitatively in capsules and oral drops by using flame atomic absorption spectrometry (FAAS). A new simple method for disrupting liposomal structure, dissolving and extracting entrapped iron salt (ferric pyrophosphate) in one step prior using FAAS, is achieved. This method is based on acidic digestion using boiling mineral acid for 15 minutes, it was also standardized by applying it on raw material (iron III- hydroxide polymaltose complex) and the iron concentration was determined by FAAS. Through results obtained in this study, liposomes were successfully digested with an accurate determination of liposomal iron. The percentage of iron concentration was between 96 to 104 % from the authorized content in capsules and oral drops. For iron complex in raw material the percent recovery was between 96 to 99.4 %. Thus, a versatile method was developed to facilitate determination of iron in liposomal dosage forms in short time, low requirements and lower costs.

Keywords: Nano-particles, Liposomal iron, Flame atomic absorption spectrometry, Acidic digestion, Mineral acid.

1. INTRODUCTION

Liposomes are one of the most common and well-investigated nano-carriers for targeted drug delivery, which attracted a wide attention in the pharmaceutical industry field [1].

Liposomes are biodegradable and generally considered to be pharmacologically in active with minimal toxicity, as they tend to be composed of natural phospholipids. The main advantage of systemic liposomes arise from their ability to reduce drug dosages due to the improved pharmacokinetic effect. So, they have lower side effects and better compliance [2, 3].

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Iron delivery via liposomes is an auspicious approach, as iron deficiency anemia (IDA) is one of the most prevalent nutritional deficiency disorders [4]. Iron salts such as ferrous fumarate, ferrous sulphate and others, are used to treat IDA. Low cost and wide availability of these oral iron salts are their key advantages. However, they have many limitations where GI intolerance (abdominal pain, constipation, black or tarry stool) is the most frequent side effect. Moreover, daily supplementation of oral iron salts increases hepcidin expression for nearly 24 hours which results in lower absorption of iron the next day [5].

Therefore, designing a new type of a stable iron supplementation with high absorption and lower side effects remains a challenging goal. So the importance of liposomal iron drugs becomes clear.

In addition, choosing a reliable method to specify

iron in liposomal dosage forms is an important issue. Several instrumental analytical techniques and different strategies for sample preparation have been used for such purpose:

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) provides the most satisfactorily sensitive and accurate technique. Unfortunately, expensive devices and strict protocols limit the prevalence of this technique in routine experiments [6]. High detection costs have also hindered the application of Inductively Coupled Plasma-Optical emission Spectroscopy (ICP-OES) and Electrothermal Atomic Absorption Spectrometry (ET-AAS), [7, 8, 9]. Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) is the appropriate atomization technique to determine concentration of iron in specimens, but its slow analysis and high cost restrict using it [10]. The photometric method is a good choice but in comparing it with Flame Atomic Absorption Spectrometry (FAAS), the latter is faster and more selective. Moreover, the determined relative standard deviations for the atomic absorption are less than those of photometric method, proving that FAAS is more accurate [11]. In addition, FAAS is one of the most practical methods with significant precision and accuracy, it is remarkable for its selectivity and speed; 10-15 seconds per specimen [12].

Choosing and applying the analytical method is not the only difficulty, sample preparation still represent the most time consuming and sensitive step. In liposomal dosage forms, it is necessary to consider disrupting the liposomal structure, dissolving and extracting the entrapped drug prior to measuring its concentration. Former methods applied on liposomal dosage forms need at least two steps for sample preparation before iron determination; one for destroying liposomes and the other for adding a material to solve and extract iron salt. Further, it is necessary to use very high speed centrifugal device or ultrafiltration technique for drug separation [13]. These former methods have many disadvantages such as many working phases, long time and more various equipment which lead to expensive costs in comparison with boiling acid disrupting technique.

Hence, based on using an acidic extraction in traditional multi-mineral preparations before measuring mineral concentrations by FAAS, this method was first applied on liposomal dosage forms containing only iron [14].

The main goal of this work is to draw attention on a very simple methodology for disrupting liposomes, solving and extracting iron salt entrapped in the liposomal structure in one step by boiling mineral acid as an alternative to surfactants or organic solvents before iron determination by FAAS.

Materials and Methods:

1. Apparatus

Flame atomic absorption spectrometry device (novAA 400, Analytic Jena), sensitive weighing scale (Kern 870) and electric heater.

2. Reagents and materials

Analytical grade concentrated acids: HCl (Chem-Lab Company, Belgium), HNO_3 (E.Merck, D-6100 Darmstadt, F.R.Germany) and $HClO_4$ (E.Merck, Darmstadt, Germany). Ferosom Forte capsules (30 mg Fe), and oral drops (7mg Fe/1 ml) of the Syrian market as a source of liposomal iron manufactured by United Pharma Canada Company which contain ferric pyrophosphate. Iron III- hydroxide polymaltose complex 34% (Chempifine Chemicals, India).

All solutions used in the experimental work were prepared by using distilled water.

3. Sample preparation and digestion procedures

Capsules; Prior to analyze three Ferosom Forte capsules, mass of the content was calculated. Total digestion of samples was carried out by mixing approximately 0.1 g of the sample with 20 ml of concentrated acid (1 M of HCl). Resulted suspension was heated for 15 minutes, after which it turned into clear

solution. Following to cooling, distilled water was added to reach 100 ml, and after an appropriate dilution, the concentration of iron was determined by FAAS.

The same procedure was repeated by changing the concentrated acid to $HClO_4$ (1 M) and HNO_3 (1 M) respectively.

Oral drops; The above mentioned steps were applied following to taking of almost 0.1 g volume of oral drops.

Iron (III)-hydroxide poly maltose complex (raw material):

A series of standards was prepared, each of different concentration of iron complex (1-3-6-10-15-30-50) mg/L. Standards were best prepared by a gradual dilution of a single stock solution.

The most concentrated standard was prepared following to taking an iron complex calculated mass, and being boiled it with concentrated HCl (1 M) for 15 minutes after which distilled water was added to reach the desired volume. A portion of the volume was diluted to prepare the next most concentrated standards, proceeding on with this dilutions process till standards were accomplished. After that iron concentration of standard solutions was determined by FAAS.

Results:

In the present work, disruption of liposomal structure and extraction of the entrapped iron salt have been accomplished in one step, using one of these boiling acids (HCl, $HClO_4$, HNO_3), and then concentration of iron was measured by FAAS. By applying this method some advantages such, low cost, high speed and low requirements are observed.

Capsules; Following to disrupting the liposomal structure via acidic digestion and the addition of distilled water to reach 100 ml, the resulted solution was diluted 10 times to be in the device measurement range where each test was repeated thrice (n=3). The resulted concentration of iron is illustrated in **Table1.**

Table1: Concentration of Fe (mg/L) in capsules containing liposomal iron, by FAAS after an acidic digestion:

Acid (1 M)	Capsule 1	Capsule 2	Capsule 3	Mean ± SD
HCl	29.15± 0.22[a]	30.53 ± 0.10[a]	29.02 ± 0.09[a]	29.57 ± 0.14[b]
$HClO_4$	29.54 ± 0.63[a]	29.9 ± 0.65[a]	29.42 ± 0.48[a]	29.62 ± 0.58[b]
HNO_3	30.57 ± 0.12[a]	30.01 ± 0.15[a]	30.1 ± 0.2[a]	30.22 ± 0.15[b]

Values are represented as the Mean ± Standard deviation. (n=3).

[a] The average for three measurements.

[b] Mean measurements in three capsules.

Oral drops; Following to disrupting liposomal structure through acidic digestion and the addition of distilled water to reach 100 ml, the resulted solution was diluted 10 times to be in the device measurement range, each test was repeated thrice (n=3). The resulted concentration of iron is illustrated in **Table2.**

Table 2: Concentration of Fe (mg/ml) in oral drops containing liposomal iron, by FAAS after an acidic digestion:

Acid (1 M)	Oral drops 1	Oral drops 2	Oral drops 3	Mean ± SD
HCl	6.96 ± 0.25[a]	7.08 ± 0.10[a]	6.85 ± 0.05[a]	6.96 ± 0.13[b]
$HClO_4$	6.74 ± 0.05[a]	7.14 ± 0.20[a]	6.93 ± 0.39[a]	7.01 ± 0.15[b]
HNO_3	6.92 ± 0.18[a]	7.27 ± 0.11[a]	7.27 ± 0.40[a]	7.15 ± 0.23[b]

Values are represented as Mean ± Standard deviation. (n=3).

[a] The average for three measurements.

[b] Mean measurements in three glasses of oral drops.

Iron (III)-hydroxide polymaltose complex; To standardize an acidic digestion method, standards containing known amounts of iron (III)-hydroxide polymaltose complex were used and their concentrations were measured by FAAS after an acidic digestion with HCl (1 M), each test was repeated thrice (n=3). The resulted concentration of iron is illustrated in **Table 3.**

Table 3: Concentration of Fe (mg/L) in standard series of iron (III)-hydroxide polymaltose complex, by FAAS after an acidic digestion with HCl (1M) :

Concentrations of standard solutions	First measurement	Second measurement	Third measurement	Mean ± SD
1 mg/L	1.002	0.98	0.98	0.99 ± 0.006
3 mg/L	2.97	2.98	2.92	2.96 ± 0.02
6 mg/L	5.72	5.83	5.76	5.77 ± 0.04
10 mg/L	9.53	9.53	9.70	9.59 ± 0.08
15 mg/L	15.03	14.76	14.94	14.91 ± 0.11
30 mg/L	29.24	29.44	29.67	29.45 ± 0.17
50 mg/L	49.24	49.47	49.38	49.36 ± 0.09

Values are represented as Mean ± Standard deviation.

To evaluate the resulted concentrations of the standard series after an acidic digestion, percentage recovery was calculated. Results are illustrated in **Table 4.**

Table 4: Percent recovery for each solution in standard series after an acidic digestion with HCl (1M) :

Concentrations of standard solutions	Recovered concentrations	Percent recovery
1 mg/L	0.99 mg/L	99 %
3 mg/L	2.96 mg/L	98.6 %
6 mg/L	5.77 mg/L	96.2 %
10 mg/L	9.59 mg/L	95.9 %
15 mg/L	14.91 mg/L	99.4 %

Concentrations of standard solutions	Recovered concentrations	Percent recovery
30 mg/L	29.45 mg/L	98.2 %
50 mg/L	49.36 mg/L	98.7 %

Discussion:

Results in **Table 1** indicate that the range of resulted concentrations of iron was between 97_102% of the authorized content in capsules (30 mg of Fe per one capsule), while in **Table 2,** it ranges between 96_104% of the authorized content in oral drops (7 mg Fe/1 ml). It is thus deduced that using boiling mineral acid can successfully disrupt liposomal structure, extract and dissolve the iron salt (ferric pyrophosphate) in one step and in a short time not exceeding 15 minutes.

Furthermore, there is no obvious difference between three acids (HCl, $HClO_4$, and HNO3) in the resulted concentrations of iron, so any of them can be used taking into consideration safety rules. It must be emphasized that the use of mineral acids in laboratories entails considerable health and safety risks, but with proper handling, the potential hazards may be mitigated.

In many previous studies [15, 16, 17], organic solvents were used to disrupt liposomal structure depending on that liposomes are made of lipids. These organic solvents are hydrophobic and incapable to solve ferric pyrophosphate after disrupting the lipid bilayer, so there will be need to use a high speed centrifuge or ultrafiltration to separate iron salt and the addition of another solvent to dissolve ferric pyrophosphate before determination of its concentration by FAAS.

Thus, it is clear that in many previous studies, many steps are needed before measuring iron concentration in liposomal dosage forms, long time and high costs. Besides, organic solvents have lower boiling points and can be easily evaporated at room temperature and badly affect our surroundings.

On the other hand, using surfactants previously [13] has also many limitations, not only their higher costs compared with mineral acids, moreover there is a need to use a centrifuge or ultrafiltration for the same purpose upon using organic solvents.

To standardize the digestion method with mineral acids, it was applied on raw material (Iron (III)-hydroxide polymaltose complex), the resulted concentrations of iron were very close to the prepared concentrations of standard series' solutions with percentage recovery between 96_99.4 %. As a result, it is confirmed that acidic digestion before measuring concentrations of iron by FAAS does not negatively affect iron with the possibility of following this method in case of iron salts or iron-saccharide complexes.

Consequently, acidic digestion enables reaching the goal of disrupting liposomal structure and solve iron salt in one step with no need of additional requirements such as centrifuge or ultrafiltration. This is positively reflected on time and costs.

Conclusion:

In this paper, an acidic digestion was performed to facilitate the quantitatively determination of iron in liposomal dosage forms. This method enabled disrupting liposomes and extracting iron salt in one step by boiling mineral acid. And it was also standardized through applying it on raw material.

Following to the acidic digestion, FAAS was applied for iron quantitative determination in raw material and liposomal dosage forms. This analytical technique is practical and not time consuming. Furthermore the operation technique is fairly easy.

Such method is a promising alternative to other methods which use surfactants and organic solvents and need two steps, one for destroying liposomes and the other for solving extracted iron salt.

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Abbreviations:

FAAS: Flame Atomic Absorption Spectrometry

IDA: Iron Deficiency Anemia

GI: Gastrointestinal

ICP-MS: Inductively Coupled Plasma-Mass Spectrometry

ICP-OES: Inductively Coupled Plasma-Optical emission Spectroscopy

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تحديد الحديد في الأشكال الصيدلانية الليبوزومية عن طريق تقنية الامتصاص الذري باللهب بعد التهضيم الحمضي

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ملخص

يشكل تحسين المطاوعة لأصناف الحديد الفموي أحد أهم الأهداف في المعالجة الدوائية، وقد قدّم تغليف الحديد ضمن جزيئات نانومترية (ليبوزومات) فرص جديدة وواعدة لتحسين التحمل ومطاوعة المريض للعلاج الفموي بالحديد. سابقاً تم استخدام العديد من التقنيات التحليلية للتحديد الكمي للحديد، في هذا العمل تم تحديد الحديد كمياً في مستحضرات صيدلانية ليبوزومية (كبسولات–نقط فموية) بالاعتماد على تقنية الامتصاص الذري باللهب. وقد تم تطبيق طريقة بسيطة وجديدة لم يتم استخدامها سابقاً لتحطيم البنية الليبوزومية المحيطة بملح الحديد، وحلّ واستخلاص الملح المتضمن ضمن البنية الشحمية (بيروفوسفات الحديد)، وذلك بخطوة واحدة قبل تطبيق تقنية الامتصاص الذري. يعتمد مبدأ هذه الطريقة على تحضير العينة باستخدام التهضيم الحمض بأحد الحموض المعدنية المركزة لمدة 15 دقيقة، وتم تقييس هذه الطريقة بتطبيقها على مادة أولية من معقد بولي مالتوز هيدروكسيد الحديد، بعد ذلك تم قياس تركيز الحديد باستخدام جهاز الامتصاص الذري. من خلال النتائج التي تم التوصل لها في هذه الدراسة، تبين أنه تم تفكيك الليبوزومات المغلفة لملح الحديد بشكل كامل باستخدام طريقة التهضيم الحمضي، وتراوحت تراكيز الحديد بعد تطبيق هذه الطريقة بين (96–104%) من الكمية المصرح بها على عبوات الكبسولات والنقط الفموية للحديد الليبوزومي، أما بالنسبة للمادة الأولية فقد تراوحت التراكيز التي حصلنا عليها بين (96–99.4%). وبذلك يمكن القول أننا توصلنا لطريقة جديدة تسهل تحضير عينة الحديد الليبوزومي قبل قياس تركيزه بجهاز الامتصاص الذري، وقد تم تحقيق ذلك خلال وقت قصير واستخدام معدات بسيطة وهذا انعكس بشكل إيجابي على الكلفة.

الكلمات الدالة: جزيئات نانومترية، حديد ليبوزومي، الامتصاص الذري باللهب، تهضيم حمضي، حمض معدني.

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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 ثلاث مرات في العام في الوقت الحالي، ومواعيد صدورها (كانون الثاني وأيار وأيلول) 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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