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***Jordan Journal of Pharmaceutical Sciences* (*JJPS*)** is a scientific peer-reviewed publication that focuses on current topics of interest in pharmaceutical sciences.

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INTRODUCTION

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

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

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

Letter from the Editor-in-Chief

Another year went by. It was an extraordinary year that none of us will soon forget, not only because of hard health times, but also because of the bad economic crisis. However, after every dusk comes the light, hoping that 2021 would be the start of the dawn. Jordan Journal of pharmaceutical Sciences (JJPS) completed 2020 publishing 4 issues on regular times; one issue per quarter (achieving an extra issue than the years before), besides having 10 articles per issue (instead of 5) in order to decrease the waiting time for the accepted articles to be published; trying to serve as much researchers as we can.

One of the achievements is the diversity areas of submissions to JJPS, the latter makes JJPS distinguished with an added value of a different taste that hopefully matches the journal readers' desires in Jordan as well as in the region. Nowadays we have submissions not only in the pharmaceutical chemistry, pharmacognosy and pharmacology, but also in pharmacy practice, clinical pharmacy, pharmaceutical care and behavioral areas related to humans and patients such as psychological considerations during the COVID-19 pandemic. Furthermore, JJPS received submissions from all around the world; giving readers the opportunity to be exposed more to different scientific research patterns worldwide with an increase in number of submissions by 62% in 2020 compared to 2019. Moreover, citations increased in 2020.

The new members in the editorial board are distinguished professors representing almost all fields of pharmaceutical sciences from different backgrounds coming from diversified research schools from USA, Canada, Europe, Australia, and Jordan. Also, they came from different work environments: governmental and private higher education institutions. The latter started smart and hard work toward becoming one of your choices to submit your article in any of the pharmaceutical fields.

In the new issues of JJPS in 2021, we will see an editorial commentary written by one of our colleagues in JJPS expressing one of the interests and thoughts related to the status que in general from their point of view.

Finally, it is really a great honor to have a new advisory board consisting of well-known scientists from different regional and international countries representing almost all pharmaceutical fields; the JJPS family is sure that the respected scientists will have a positive impact and will add value particularly in the quality of manuscripts accepted for publication. Looking forward to more achievements in 2021.

Prof Ibrahim Alabbadi
Editor-in-Chief

Editorial Commentary

Dear Colleagues and Researchers,

As one of the Editorial Board members of the Jordan Journal of Pharmaceutical Sciences (JJPS), it is my pleasure to write for all my colleagues in the field of pharmaceutical analysis. Although JJPS is a peer-reviewed journal for original research articles, review articles, and short communications related to all aspects of pharmaceutical sciences, I am encouraging you to submit your most recent advances and research work in pharmaceutical and biomedical analysis fields, including drug analysis, analytical methodology, quality control, and instrumentation.

JJPS is indexed in Scopus (Elsevier), Ulrich's Periodicals Directory, Google Scholar, and EBSCO. Furthermore, JJPS has an open access policy, which enables your research to become available to all scientists in the field and thus, increases exposure and citation.

It has to be mentioned that the journal is overseen by an Editorial Advisory board, which consists of eminent and competent researchers in all fields of pharmaceutical sciences. In addition, the editorial board members have rich academic qualifications. Therefore, with the network of both boards, the widespread of the journal is distinguished.

At the end, I encourage all scientists involved in the pharmaceutical analysis field to submit their work, or their postgraduate students work and sense the professionalism that they will encounter from JJPS editorial staff and board. At JJPS, we believe in a quick scientific reviewing process since responding to authors promptly is ideal for pursuing widespread scientific knowledge.

Hoping for more collaborations between us in the future.

Professor Wael Abu Dayyih
Department of Pharmaceutical Medicinal Chemistry and Pharmacognosy
Faculty of Pharmacy and Medical Sciences
University of Petra
wabudayyih@uop.edu.jo

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The bleeding and clotting time analysis of the stem extract of *Musa paradisiaca var. sapientum (L.) Kunze* on hemostatic response

Hendrik Setia Budi[1*], Wisnu Setyari Juliastuti[1], Yuniar Putri Sulistyowati[1]

[1]Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga
**Correspondence : Hendrik Setia Budi, Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga. Jl. Prof. DR. Moestopo No 47 Surabaya, Indonesia, 60132.*

ABSTRACT

A herbal medicine that can be used to accelerate bleeding is the Ambonese banana stem sap (Musa paradisiaca var. Sapientum) through secretion of Adenosin Diphosphate (ADP) and von Willebrand Factor (vWF) in the process of platelet adhesion on the injury vessels. The aim of this study to test the influence of Ambonese banana stem extract in a concentration of 25%, 50% and 100% against hemostatic response on mice. 24 animal study were randomly assigned into four groups. Group 1 was the control, group of 2 was given 25% extract concentration, group of 3 was given 50% extract concentration and group of 4 was given 100% extract concentration of Ambonese banana stem per-oral. Then all the groups were examined their bleeding and clotting time. The administration of Ambonese banana stem extract at the concentrations of 25%, 50 %, and 100% significantly shortened both bleeding time and clotting time compared to control group ($p = 0.00$). The group with 100% extract of the Ambonese banana stem having the shortest bleeding time and clotting time. The conclusions of this study showed Ambonese banana stem extract has potential as the hemostatic agent by shortening bleeding and clotting time on mice.

Keywords: Ambonese banana stem extract, bleeding time, clotting time, hemostatic.

1. INTRODUCTION

The surgical procedures come with a risk of complications which may include pain, nerve injury, swelling, excessive bleeding (hemorrhage), and infections. Severe intraoperative or postoperative hemorrhage is one of the few life-threatening complications for which a dentist may have to initiate management.[1,2] The bleeding disorder, it doesn't clot fast enough. This results in too much bleeding or long-lasting bleeding, and become port de entry of infection.[3,4]

Hemostasis is a complex process that leads to the formation of a blood clot at the site of vessel injury and three phases can be distinguished by vascular spasm, formation of a platelet plug and coagulation. Primary hemostasis starts immediately after damage of the vessel wall with vasoconstriction as a result of local contraction of vascular smooth muscle cells.[5] The von Willebrand factor (VWF) binds to the exposed subendothelial collagens. Platelets are tethered to the site of endothelial cell injury through the binding of VWF to the glycoprotein Ib receptor of the platelet.[6]

In major oral and maxillofacial surgical procedures, electrocautery and suture ligatures are most commonly used to control bleeding from small and major vessels. However, when the use of pressure is not effective, the use of electrosurgical instruments could endanger teeth or nerves, systemic and, or topical hemostatic agents may be needed.[7,8] The study shown the effectiveness of Plasma Rich in Growth Factors (PRGF) in controlling bleeding after dental extractions in patients with haemophilia. In this fascinating study there were no risks related to the use

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of blood derivatives, yet good haemostasis and healing of soft tissues were achieved. In other words, the post-extraction socket healed better and faster which can prevent further bleeding.[9]

Indonesia is a rich-tropical country in natural resources with the second highest biodiversity in the world after Brazil. Natural resources, such as plants and animals in Indonesia, are very varied, which can be used for improving natural products. The importance of biodiversity, e.g., in traditional medicine and agriculture, is deep-rooted in Indonesian society.[10]A plant that can be efficacious as a drug and has long been used to accelerate wound healing and tooth pain relief by the people of the village of Trunyan, Bali is Ambonese banana stem extract.[11] In banana plants, there are significant compounds, such as saponins, flavonoids, anthraquinones, and tannins contained in both its fruit and sap, as well as lectin compounds found in banana stems. Lectin plays a role in stimulating mitotic cells which has an effect on accelerating wound healing and bleeding cessation.[12,13] Meanwhile, tannins are astringent, stopping bleeding in the wound.[14] This study aimed to prove Ambonese banana stem extract (Musa paradisiaca var. Sapientum) administration can accelerate wound healing through hemostatic response.

Materials and Methods

Plant material and methods

Collected from the Plant Conservation Center Botanical Gardens Purwodadi, Pasuruan, Indonesia, Ambonese banana tree will be able to be harvested at age 12-13 months, with the height is about 2.5-3 m, and the stem diameter is about 17.3-18.9 cm wide. Determination was carried out by the Indonesian Institute of Sciences (LIPI) Purwodadi, Pasuruan, Indonesia number 1036/IPH.UPT.03.4/HM/IX to prove that the type of banana tree used is a type of Ambonese banana.

Preparation of the sample

To get Ambonese banana stem's sap in large quantities, the stem need to be cut at the lower end part, then clean it to remove the impurities. After that, made it into small pieces weighing 200 grams, add 200 ml of sterile distilled water and blend until smooth. The sap and water that have been mixed together are then filtered using a Buchner funnel connected with a vacuum pump (Gast, USA brand) with whatman filter paper number 1. The filtration results are dried using a freeze dryer so that it can be stored for a long time. The making of Ambonese banana stem extracts with concentration of 25%, 50% and 100% was carried out by dissolving dried preparations in sterile aquadest.

Experimental design

The ethical approval to conduct the study was taken from the Research Ethics Committee, Faculty of Dentistry, Universitas Airlangga. This research used a randomized post-test only control group design. We used 24 male mice divided into 4 groups. The first group was the control group with the administration of vehicle. The second group was given Ambonese banana stem extract at a concentration of 25%. The third group was given Ambonese banana stem extract at a concentration of 50%. The fourth group was given Ambonese banana stem extract at a concentration of 100%. Those extracts were given for 3 consecutive days. Bleeding and clotting time examinations then were conducted in each group.

Bleeding time experimental

The mice (*Mus muscullus*), male, 20-30 gram of body weight were anesthetized with diethyl ether using inhalation method. Mice are put in a transparent box for easy viewing, which contains cotton with ether, and within a few seconds the activity of the mice begins to decline and then falls asleep. The mice's tail was cut 3 cm long from the tip of the tail, blood that came out after cutting was dropped on absorbed paper. The stopwatch was started to run along with the visible blood coming out of the mice that had been cut. Blood dripped on absorbent papers that should not touch the wound. The stopwatch was then stopped when blood did not drip anymore on the absorbent papers.[15] Mice are generally feed containing low fiber (5%), protein (20%) and fat (5-10%) in pellet.

Clotting time experiment

Blood was taken from the heart of anesthetized mice by surgery using a scalpel, and then 1cc of blood was taken using a tuberculin syringe directly from the heart. The stopwatch was started to run along with the entry of blood on the syringe. The blood that had been taken was then inserted into capillary tube to measure the clotting time. Afterwards, the tubes were tilted slowly and re-stood up at 10-second intervals until the blood in the tubes did not flow again when they were tilted to a tilt angle of 90°. The stopwatch was stopped when the blood had not flowed even though the tubes were tilted at an angle of 90°. Surgical wounds then were stitched and oral antibiotics and analgesics were given.[16]

Statistical analysis

All the data were tabulated and assessed using the Statistical Package for Social Sciences (SPSS) version 20.0. Comparison of various time among four study groups were done using ANOVA test and post hoc Tukey test. A p-value ≤ 0.05 was considered to be statistically signifiant.

Results

Phytochemical components

The Saponin, flavonoid, tannin, anthraquinone and lectin levels were analyzed using UV-Visible Spectrophotometer by comparing the absorbance values of standard compounds with the extract of ambon banana stem sap. Detection of saponin compounds with a wavelength of 215 nm, flavonoids with wavelengths of 226 nm, tannins with wavelengths of 275 nm, anthraquinones with wavelengths of 285 nm and lectins with wavelengths of 228 nm (see table 1).

Table 1. The concentration of each component in Ambonese banana stem extract

Components	*Number of replicant*	*Concentration (mg/100mL)*
Saponin	*3*	*1.30*
Flavonoid	*3*	*0.28*
Tannin	*3*	*1.50*
Antraquinone	*3*	*0.30*
Lectin	*3*	*0.11*

Hemostatic properties of plant extract

Bleeding time is a clinical laboratory test performed to evaluate platelet function. It involves the creation of a standardized incision and timing the cessation of bleeding. At sites of vascular injury, platelets are recruited to exposed subendothelial extracellular matrix components via specific platelet receptors involved in adhesion and aggregation. The herbal medicines contain a lot of different compounds which some of them have great complexities. Plants substances such as polysaccharides, mucilages and tannins may modulate and modify the effects of "active components".

Results of the One-way ANOVA test showed that the administration of Ambonese banana stem extract at the concentrations of 25%, 50 %, and 100% significantly shortened both bleeding time and clotting time compared to control group ($p = 0.00$). Ambonese banana stem extract at the concentrations of 100% even had the shortest bleeding time compared to the other groups. In other words, the higher the concentration is, the shorter the bleeding time is. Similarly, in the clotting time examination, the group given Ambonese banana stem extract at the concentration of 100% had the shortest clotting time compared to the other groups (see table 2).

Table 2. Bleeding and clotting time test

Groups	$\bar{X} \pm$ SD	
	Bleeding Time **(second)**	***Clotting Time*** **(second)**
1	$149.5^{a} \pm 33.2$	$183.3^{a} \pm 19.5$
2	$75.2^{b} \pm 23.2$	$128^{b} \pm 25.8$
3	$53.2^{c} \pm 7.9$	$94.7^{c} \pm 17.1$
4	$35^{d} \pm 8.53$	$86.33^{c} \pm 13.2$

The values with different superscript letters in a column are significantly different ($p<0.05$).

Discussion

The results of this research showed that bleeding time and clotting time in Group 1 (control) had a significant difference when compared with Group 2 (given Ambonese banana stem extract at the concentration of 25%), Group 3 (given Ambonese banana stem extract at the concentration of 50%) and Group 4 (given Ambonese banana stem extract at the concentration of 100%). In other words, the administration of Ambonese banana stem extract at the concentrations of 25%, 50%, and 100% can accelerate both bleeding time and clotting time in mice.Moreover, it is also known that Group 1 needed the longest bleeding time compared to the other groups (see figure1). Ambonese banana stem extract actually contain various active compounds, namely lectins and tannins.[17,18]

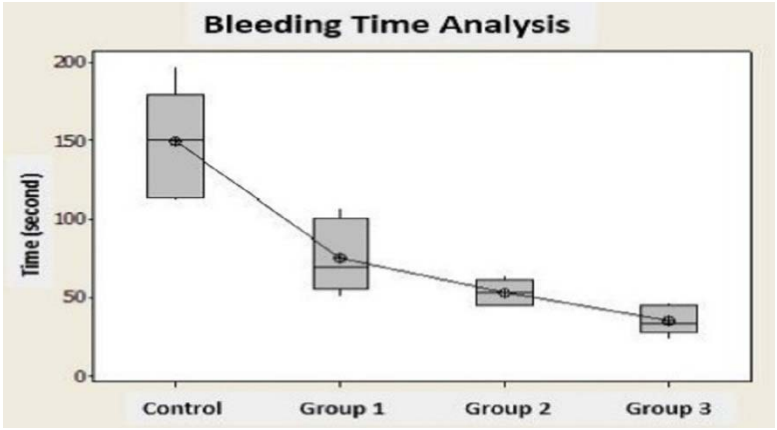

Figure 1. Examination of bleeding time by cutting the mice's tail.

C-type lectins are a subgroup of lectins, carbohydrate-recognizing proteins, which recognize and bind carbohydrates in a Ca2+ ion-dependent manner. They play a crucial role in hemostasis, a complex system to stop bleeding, which encompasses coagulation, platelet activation, and thrombus formation.[19] The mechanism of lectin in accelerating the process of hemostasis is through some stages.[20] After an injury to a blood vessel, platelets normally in a state of silence will turn into active and move towards an injured blood vessel to form a platelet plug at aninitial phase. This phase is called platelet adhesion, which is the attachment of platelets to injured tissue.[21-23]

Tannic acid is a commercial compound which is similar to the plant polyphenol tannin, that stops bleeding from mucous membrane via vasoconstriction.[2] Tanin is another active compound contained in Ambonese banana

stems playing a role in increasing ADP secretion in blood. ADP compound is a vasoactive chemical substance released by the body during vascular injury. ADPs and other chemicals, including serotonin, then trigger platelets to deform irregularly and become sticky with each other. Thus, one platelet and another will be easier to bind. An increase ADP levels in the blood then will result in high platelet aggregation so that platelet plugs as a mechanism for body hemostasis will soon be formed and will accelerate the bleeding time indicated in this research by the rapid cessation of blood coming out of the mice that were cut off.[24]

Ambonese banana stem extract at the concentration of 100% is thought to have higher active compounds of tannin and lectin than concentrations of 25% and 50%. This high level of the active compounds, tannins and lectins, can increase platelet activity to adhesion and aggregate platelets when a vascular injury occurs. This is in accordance with the results of this research that showed that the group of mice with Ambonese banana stem extract at the concentration of 100% had the shorten bleeding compared to the other groups.

In addition to accelerating the bleeding time, the administration of Ambonese banana stem extract at the concentrations of 25%, 50% and 100% can also accelerate the process of clotting time in mice (see figure 2). According to Ogle (2008), clotting time is an ability of blood to change from liquid to semi-solid. After the platelet plug is formed, the blood clotting cascade begins.[7] This process runs through extrinsic and intrinsic blood clotting pathways involving 13 blood clotting factors. After the process, a prothrombin activator is formed which has a task of converting prothrombin to thrombin. Next, thrombin converts soluble fibrinogen into solid fibrin polymer threads. These fibrin threads will later become nets that will capture plasma, blood cells, and platelets to become a clot.[9,25]

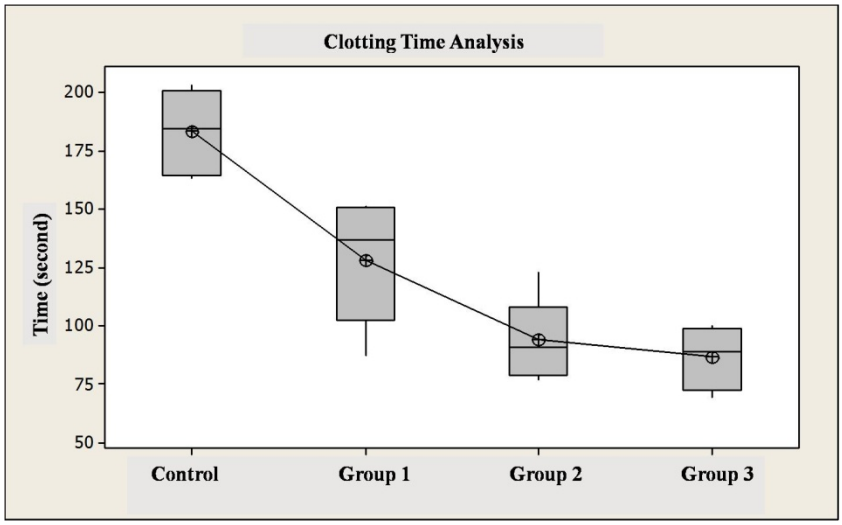

Figure 2. Examination of clotting time with modified Lee and White tube methods.

High platelet aggregation activities, furthermore, were found in the group with the administration of Ambonese banana stem extract since the effects of the active compounds, tannins and lectins, will be directly proportional to the increased clotting time in mice. The solid fibrin threads formed then will attract more platelets carrying out more aggregation. As a result, the blood clotting process will occur faster. The results of this

research are the same as those reported by Bamidele, et al. (2010), examining the effects of methanol ageratum conyzoides leaf extract on hemostatic responses in mice. The research conducted by Bamidele, et al. (2010), finds that a decrease in bleeding time will compensate for a decrease in clotting time in mice.[26]

Based on the previous hypothesis, the higher the concentration of Ambonese banana stem extract is, the higher the platelet aggregation activity occurs. If more platelets are attached to one another, then the fibrin threads that have formed in the initial phase of blood clotting will attract more thrombocytes, and blood clots will also occur faster. But, the decrease in clotting time in the group with Ambonese banana extract at the concentration of 100% was not significantly different from the group with Ambonese banana extract at the concentration of 50%. Even though at the concentration of 100% had the fastest clotting time compared to the other groups.However, based on the results of Duncan's distance test, Ambonese banana extract at the concentration of 50% did not differ significantly compared to that at the concentration of100%.

This may occur due to the influence of blood clotting factors working on blood clotting cascade. Before the formation of fibrin threads which will later capture blood cells and plasma, blood clotting cascade occurs first, affected by various factors including 13 intrinsic and extrinsic blood clotting factors, such as fibrinogen, prothrombin, thromboplastin tissue, calcium, and vitamins K. In this study, unfortunately, there was no observation on the blood clotting factors. Hence, it is assumed that there was less significant difference between the group with the administration of Ambonese banana stem extract at the 100% and the group with that at the concentration of 50%. This finding may be influenced by the blood clotting factors.

Finally, based on the results, it can be said that the study hypothesis has been proven. The administration of Ambonese banana stem extract has an effect on accelerating the hemostasis response in mice characterized by an increase in both bleeding time and clotting time. The active compounds of tannins and lectins working synergistically in mice can lead to an effect on accelerating both bleeding time and clotting time.

Indonesia is a rich-tropical country which plants are used as an alternative medicines. However, deeper research needs to be done to prove the efficacy of drugs from plants in order to be used by the community. Although this research has proven the benefits of Ambonese banana stem sap as hemostatic, the platelet activity on the wound needs to be known to support this study.

The use of plant-derived medicines continues to be developed through biomolecular research so that they can be used as a standardized medicine. Ambonese banana stem has the potential to be developed into hemostatics through several studies that have been done before, and even has been patented for cancer therapy. Topical and hemostatic drugs are also capable for tooth extraction or scaling and root planning in gum disease that are needed for patients with blood disorders, such as hemophilia, which are still common in Indonesia, even in the world.

Conclusions

Ambonese banana stem extract (Musa paradisiaca var. Sapientum) have a potential as hemostatic agent by shortening bleeding and clotting time on mice.

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تحليل زمن النزف والتخثر لمستخلص ساق *Musa paradisiaca var. sapientum* (L.) *Kunze* على الاستجابة المرقئة

هندريك بودي، ويسنو جولياستوتي، يونيار سوليستيواتي*

*كلية طب الأسنان،جامعة ايرلانجا

ملخص

أحد الأدوية العشبية التي يمكن استخدامها لتسريع النزيف هو عصارة جذع الموز الأمبونية **(Musa paradisiaca var. Sapientum)** من خلال إفراز **Adenosin Diphosphate (ADP)** وعامل **von Willebrand (vWF)** في عملية التصاق الصفائح الدموية على الأوعية المصابة .تهدف هذه الدراسة إلى اختبار تأثير مستخلص جذع الموز الأمبوني بتركيز **25** ٪ و **50**٪ و **100** ٪ استجابة مرقئ على الفئران .تم تقسيم **24** دراسة حيوانية بشكل عشوائي إلى أربع مجموعات .المجموعة الأولى كانت المجموعة الضابطة ، المجموعة الثانية أعطيت**25** ٪ تركيز المستخلص ، المجموعة الثالثة أعطيت تركيز المستخلص **50**٪ والمجموعة الرابعة أعطيت تركيز **100** ٪ من مستخلص جذع الموز الأمبوني عن طريق الفم .ثم تم فحص جميع المجموعات وقت النزيف والتجلط .أدى إعطاء مستخلص جذع الموز الأمبوني بتركيزات**25** ٪ و **50** ٪ و **100** ٪ إلى تقصير وقت النزف ووقت التخثر بشكل كبير مقارنة بمجموعة التحكم **(p = 0.00)**. المجموعة التي تحتوي على مستخلص**100** ٪ من جذع الموز الأمبوني لها أقصر وقت للنزيف ووقت التخثر .أظهرت استنتاجات هذه الدراسة أن مستخلص جذع الموز الأمبوني لديه القدرة على أن يكون عامل مرقئ عن طريق تقصير وقت النزف والتخثر على الفئران.

الكلمات الدالة: مستخلص جذع الموز الأمبوني ، زمن النزف ، زمن التخثر ، مرقئ.

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Effects of Selected Malaysian Kelulut Honey on Biofilm Formation and the Gene Expression Profile of Staphylococcus Aureus, Pseudomonas Aeruginosa and Escherichia Coli

Mohammad A. Al-kafaween[1], Abu Bakar Mohd Hilmi[1]*, Hamid A. Nagi Al-Jamal[1], Norzawani Jaffar[1], Hiba Al-Sayyed[2], Mohd Khairi Zahri[1]

[1]Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia.

[2]Department of Nutrition ,Faculty of Pharmacy and Medical Sciences, University of Petra, Amman ,Jordan

[*]Corresponding Author: Abu Bakar Mohd Hilmi

Email: mhilmiab@unisza.edu.my

ABSTRACT

Honey is now being renowned as an alternative treatment due to its broad-spectrum antibacterial activity and the inability of bacteria to develop resistance after exposure to it. Honey has been shown to be bactericidal against *E. coli, S. aureus* and *P. aeruginosa* as it destabilizes the bacteria's cell wall. This study was designed to evaluate the effect of a Malaysian *Kelulut* honey on established biofilm, prevention biofilm and on level of gene expression in *S. aureus*, *P. aeruginosa* and *E. coli*. Established biofilm and prevention biofilm assays were conducted on three strains of *S. aureus*, *P. aeruginosa* and *E. coli* using 96-well plates with five different concentrations of honey namely 5%, 10%, 20%, 30%, and 40% (w/v) and the level of gene expression assay was conducted using RT-qPCR. *Kelulut* honey was able to reduce the biofilm mass formation up to 39%, 41% and 37% in *S. auerus*, *P. aeruginosa* and *E. coli* respectively. The lowest concentration of *Kelulut* honey found to prevent biofilm formation in *S. auerus*, *P. aeruginosa* and *E. coli* was 30% (w/v). Approximately fourfold reduction in the total number of viable bacterial cells of *S. auerus*, *P. aeruginosa* and *E. coli* was observed following treatment with 40% (w/v) Kelulut honey. The RT-qPCR showed that twelve genes including *argF, purC, adh, fabG, fliA, fliC oprB, oprH, yjfO (bsmA), ycfR (BhsA), lsrA* and *tnaA* were downregulated, whilst, eight genes including *scdA, pyk, menB, oprC, lasR, algU, rpoS* and *evgA* were upregulated after exposure to *Kelulut* honey. This study showed the efficacy of Kelulut honey against biofilm, and that different concentrations of honey possess different degrees of potential effect on established biofilm. Also, a decreased expression of virulence genes in these bacteria will impact their pathogenicity.

Keywords: *S. aureus*, *P. aeruginosa, E. coli*, Gene expression, Malaysian *Kelulut* honey, Biofilms.

1. INTRODUCTION

All over the world, deaths from infectious diseases amounts to more than 17 million each year and most of these deaths have been linked to bacterial infections [1, 2]. Bacterial infections are generally treated with antibiotics but considering that microorganisms are known to develop numerous mechanisms of resistance [3, 4], antibiotics are losing their effectiveness rapidly [5]. Also, it is more difficult to treat the diseases caused by antibiotic resistant bacteria as opposed to treating the diseases caused by non-resistant ones [6]. Antibiotic resistant bacteria have been spreading worldwide and this has led to the increase in medical costs, hospital stays and death cases [7-9]. For this reason, recent studies are looking into the use of alternative antimicrobial

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strategies including the utilization of plants and honey products in the treatment of bacterial infections [10-13].

Among the oldest and highly reputable traditional medicines is honey, and the long history has seen the application of this substance in treating a number of human diseases [14-16]. The emergence of "apitherapy," which comprises the application of honey and other products associated with bees in treating ailments [17, 18], have preserved the positive reputation of honey. This reputation has continued up to the present day, leading to the emergence of a relatively new branch of alternative medicine, called "apitherapy", which focuses on medical applications [19, 20] of honey and other bee products [21-24]. Nowadays, different types of honey have been used in many countries as an alternative to pharmaceutical products for treating contaminated, infected, and burn wounds [20, 25-27]. This is attributed to the effectiveness of these honeys in inhibiting or killing a broad spectrum of bacteria [28- 30].

Honey comes in various kinds and in many countries, honey has been used as an alternative to pharmaceutical products particularly in the treatment of contamination, infection, and burn wounds [31, 32]. The use of honey has been factored by its ability in inhibiting or destroying a broad spectrum of bacteria [28, 33]. Accordingly, several studies have been carried out in understanding the impacts of honey on bacterial structures [34-40], but most have been focusing on Manuka honey only and mainly on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It is hence necessary to examine other types of honey such as *Kelulut* honey and other microorganisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus Escherichia coli.*

For *Kelulut* honey, its bactericidal effect on *S. aureus*, *P. aeruginosa* and *E. coli* has been investigated *in vitro*. From the electron microscopy, it appears that *Kelulut honey* deformation the cells and the cells appeared curved, distorted and the cell density was markedly decreased [40-42]. The antimicrobial activity of honey may be attributed to several factors, including high osmolarity, acidity, in addition to the presence of hydrogen peroxide (H_2O_2) and non-peroxide components, such as methylglyoxal [42-47]. In addition to exerting direct antimicrobial effects, some honey varieties have been implicated in the differential expression of a number of genes essential for bacterial survival and virulence, including those involved in stress tolerance [48], virulence factor production [49], as well as multicellular behaviors, such as biofilm formation [50], and quorum sensing [51].

Several studies have addressed different aspects of *Kelulut honey* varieties, including their physicochemical properties , their chemical composition [52-55], their antibacterial and antibiofilm activities [40, 56], and their therapeutic usefulness [42, 57, 58]. However, it is not yet known whether these anti-biofilm activities, as well as any possible anti-quorum sensing and anti-virulence activities possessed by this honey could be attributed to alteration of bacterial gene expression. [59-63]. Therefore, The effects of Kelulut honey on gene expression in *S. aureus, P. aeruginosa* and *E. coli* have not been empirically studied in the past, and for this reason, the examined genes were chosen following the published expression profiling studies, whereby the cells of *E. coli, S. aureus* and *P. aeruginosa* have been treated with honeys different from those tested in the present study. As such, these genes were chosen based on the genes involved in the generation of biofilm, quorum sensing, motility and stress survival in these test organisms. Accordingly, six genes of *E. coli* (*yjfO (bsmA), ycfR (BhsA), tnaA, lsrA, evgA*, and *rpoS*), seven genes of *S. aureus* (*argF, purC*, *adh, fabG*, *scdA*, *pyk* and *menB*), seven genes of *P.aeruginosa* (*fliA*, *fliC, oprB*, *oprH*, *oprC, lasR* and *algU*) were chosen for the expression analysis. As shown by past studies, the exposure to honey downregulated and upregulated the differential gene expression involved in biofilm construction, quorum sensing, motility and stress endurance in these test organism [59-66].

MATERIALS AND METHODS

Bacterial strains and culture conditions

Three strains of *S.auerus* (ATCC 25923), *P.aeruginosa* (ATCC 27853) and *E.coli* (ATCC 25922) were used throughout the study. Cultures of bacteria were supplied by Microbiology Laboratory, University of Sultan Zainal Abidin (UniSZA). The supplied bacteria were reconstituted into sterile Mueller Hinton Broth (MHB) (Oxid, UK) and incubated at 37°C. After 24 hours, they were sub-cultured on Mueller Hinton Agar, (MHA) (Oxid, UK) and incubated again at 37°C for another 24 hours before being processed for long storage at -80° in eppendorf tube containing MHB, and 15% glycerol. Working bacterial culture was prepared by inoculating a loopful of primary culture from -20° storage into Erlenmeyer flask containing 15 ml of MHB broth [60-61]. The inoculum was incubated at 37°C for 24 hours. After incubation time, the inoculum was incubated at 37°C for 24 hours. Then, the suspension was adjusted to be equal to 0.5 McFarland standard [62, 67].

Honey samples

Kelulut honey samples were purchased from a bee farm located in Kelantan, a state in Malaysia. All honey samples were stored in airtight amber glass bottles and stored at room temperature until further analysis [52].

Established biofilm reduction assay

Bacterial cultures were adjusted to 0.5 McFarland standard as previously described. Two hundred microliter of the culture was transferred into wells of 96-well plates and the plate was incubated for 48 hours at 37°C without shaking. Wells containing only bacterial culture served as positive control and wells containing honey only served as corresponding negative control. After 48 hours of incubation, planktonic cells were removed and then 200 µl of different concentrations of 40%, 30%, 20%, 10% and 5% (w/v) of honey were added to the wells and then the wells were incubated overnight. After incubation, the plate was washed with PBS. The plate was fixed with 2 ml µl of 2.5% glutaraldehyde for 10 minutes. Then, the plate was washed with PBS. The attached cells or biofilm was then stained with 200 µl of 0.1% crystal violet for 15 minutes and washed two times with PBS. Absorbance was determined at 540 nm wavelength using microtitre plate reader (Tecan Infinite 200 PRO, Austria). This assay was repeated in triplicate [68-71]. The reduction of biofilm mass was calculated following the formula shown below:

$$\text{Biofilm (\%)} = \frac{\text{OD (positive control)} - \text{OD (treatment)}}{\text{OD (positive control)}} \times 100\%$$

Biofilm Prevention assay

To determine the concentration of *Kelulut* honey required to prevent a biofilm of *S. aureus, P. aeruginosa* and *E. coli* and forming *in vitro,* a range of concentrations; 40%, 30%, 20%, 10% and 5% (w/v) of *Kelulut* honey were freshly prepared in Muller Hinton Broth (MHB; Oxoid, UK) from a stock solution of 50% (w/v) honey. Approximately 200µl of diluted honey was dispensed into wells of 96-well plates and inoculated with 200µl of a diluted overnight culture of the test organism (population density of 2.5×10^8 colony forming units (cfu/ml). Wells without inoculum served as a corresponding negative control and wells without added honey used as a positive control. The plates were incubated for 48 hours at 37°C and the extent of biofilm formed was evaluated by determining optical density (at 540nm) using microplate reader (Tecan Infinite 200 PRO, Austria) [70, 72]. The experiment was conducted at least three times.

Determination of biofilm viability (by total cell count)

To determine the effect of *Kelulut* honey on biofilm viability, the liquid phase from 48 hours biofilm formed in wells was discarded and contents were washed with 300µl sterile maximum recovery diluent (MRD, Oxoid, UK) to remove planktonic cells. A further 200µl of MRD was added to the washed biofilm and a sterile pipette tip was then used to scrape the bottom of the well to release adherent biofilm. The total viable count of the resulting suspension was determined using the surface drop count [73]. Diluted suspensions were plated onto nutrient agar (NA; Oxoid, UK). The plates

were and incubated for overnight at 37°C and cfu per well was calculated [52, 68, 74].

Extraction of RNA from *S. aureus, P. aeruginosa* and *E. coli* for RT-qPCR

Initially, the inoculum was adjusted as described previously, and 100 µL of inoculum was pipetted into microtiter plate with 100 µL of 20 % (w/v) concentration of honey. Meanwhile, wells with inoculum only without honey was used as a positive control. Then, the plate was incubated for 48 hours at 37ºC. After that, 1 ml of samples, treated and untreated, were re-suspended in PBS and vortexed for 3 minutes and centrifugated at 3,500g for 5 minutes. The supernatant was discarded and the pellet was washed again with PBS. Total RNA from treated and untreated sample was extracted using kit SV Total RNA Isolation System (Promega, UK). Total RNA concentrations was examined using Implen NanoPhotometer® NP80. Total RNA was converted to cDNA following the manufacturer's instructions kit (Promega, UK). For each reaction, qPCR mastermix was prepared by following the manufacturer's instructions (Promega, UK) and PCR primers were used as shown in Tables 1, 2 and 3. The following PCR protocol was used: denaturation at 95ºC for 2 minutes in one cycle, amplification at 95ºC for 15 seconds in 40 cycles and a final elongation annealing at 60ºC for 1 min in 40 cycles. Densitometry was performed using the Applied Biosystems Step One Software v2.3. To determine and calculate the level of gene expression, a modified $2^{-\Delta\Delta}$ Ct method was used [60-62, 70]. The experiment was performed in triplicate.

Statistical analysis

Data were expressed as mean ± standard deviation. Independent student's t-test from SPSS version 20 was used to compare between honey-treated and control groups. The significance level was set at $P<0.05$.

RESULTS

Established biofilm reduction assay

In general, honey samples were able to reduce the mass of established biofilm on *S.auerus*, *P.aeruginosa* and *E.coli*. The data show that different concentrations of honey sample produced different degrees of inhibitory effects on different strains of bacteria. Figure 1 shows that 40% (w/v) concentration of *Kelulut* honey was the most effective to reduce the biofilm mass of *S. auerus* (39%), *P. aeruginosa* (41%) and *E. coli* (37%).

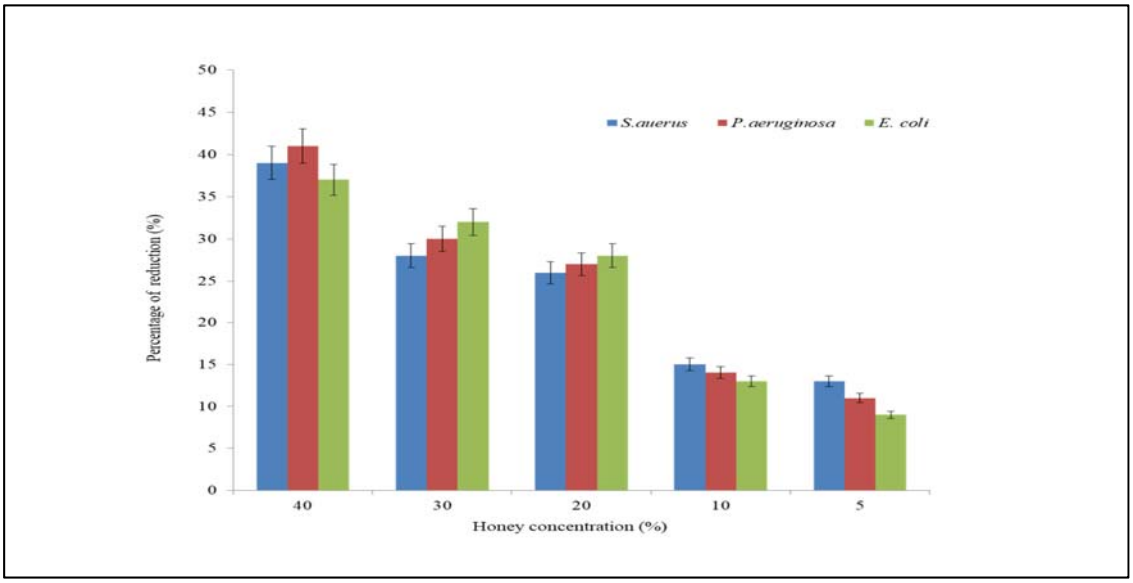

Figure 1: Percentage of reduction of *S.auerus*, *P.aeruginosa* and *E.coli* biofilm mass after exposure to *Kelulut* honey

Table 1: Gene specific primers of *S.auerus* used for RT-qPCR analysis [62]

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
argF	143	Forward	CCAAGCAGAATTCGAAGGA
		Reverse	GGATGCGCACCTAAATCAAT
purC	117	Forward	GAAGCGCATTTTCTCAACAA
		Reverse	CCCTTACCTGCCATTGTGTC
adh	124	Forward	GTTGCCGTTGGTTTACCTGT
		Reverse	TTCAGCAGCAAATTCAAACG
scdA	132	Forward	CGAAAGCAGCGGATATTTTT
		Reverse	GCGAACCTGGTGTATTCGTT
pykA	126	Forward	TGCAGCAAGTTTCGTACGTC
		Reverse	GGGATTTCAACACCCATGTC
menB	109	Forward	CTGGGGAAGGTGATTTAGCA
		Reverse	ACCGCCACCTACAGCATAAC
fabG	122	Forward	CCGGGACAAGCAAACTATGT
		Reverse	CCAAAACGTGCTAACGGAAT
*yqiL**	125	Forward	GACGTGCCAGCCTATGATTT
		Reverse	ATTCGTGCTGGATTTTGTCC

* *yqiL* was used as a reference gene for *S.aerus*

Table 2: Gene specific primers of *P.aeruginosa* used for RT-qPCR analysis [61], [66]

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
oprB	140	Forward	TGACGACGACAAGACAGGAC
		Reverse	GGTCGTTGGAAAGGTTCTTG
oprC	105	Forward	GCCTGAACATCCTCACCAAC
		Reverse	CGGTGAGCTTGTCGTAGGTT
oprH	102	Forward	CTCGACAAGGTGATCGACAA
		Reverse	GGTGTCGGAGATGTTCTCGT
fliA	192	Forward	CTCCAATTGAGCCTCGAAGA
		Reverse	TTCGTTGTGACTGAGGCTGG
fliC	121	Forward	GCTTCGACAACACCATCAAC
		Reverse	AGCACCTGGTTCTTGGTCAG
lasR	129	Forward	CGGTTTTCTTGAGCTGGAAC TCGTAGTCCTGGCTGTCCTT
		Reverse	
algU	113	Forward	GCGAGTTCGAAGGTTTGAGT
		Reverse	CTGCAGAGCTTTGTCGATTG
*rpoD**	146	Forward	GCGACGGTATTCGAACTTGT
		Reverse	CGAAGAAGGAAATGGTCGAG

**rpoD* was used as a reference gene for *P.aeruginosa*

Table 3: Gene specific primers of *E.coli* used for RT-qPCR analysis [60]

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
yjfO (bsmA)	76	Forward Reverse	CGCCAGTAACGGACCATC GTGCTTACGCTACCTATTCG
ycfR (BhsA)	81	Forward Reverse	CGAAGTTCAGTCAACGCCAGAAG TCCAGCGATCCCAGATTTGTCC
tnaA	174	Forward Reverse	CTGGATAGCGAAGATGTG CGGAATGGTGTATTGATAAC
evgA	155	Forward Reverse	TAGCGGAGACGATAATAATAATTC GTTGACTGAAGGCGGAAG
rpoS	199	Forward Reverse	CTCAACATACGCAACCTG GTCATCAACTGGCTTATCC
lsrA	178	Forward Reverse	TACTCATAACCTTCGTGGATTCTG TACTTGCGGCGAGGCTTC
*ftsA**	152	Forward Reverse	GAAGAAGTGACGCAAGAAGATG ACGCCCGAAAGTCCTACC

* *ftsA* was used as a reference gene for *E.coli*

Biofim prevention after treatment with *Kelulut* honey

The lowest concentration of *Kelulut* honey that prevented *S.auerus*, *P.aeruginosa* and *E.coli* forming a biofilm *in vitro* was found to be 30% (w/v). Inhibitory effects are normally expressed as the MIC; here this was determined by assessing optical density. All methods gave similar endpoints, although slighter wider variation was seen by different species (Figure 2).

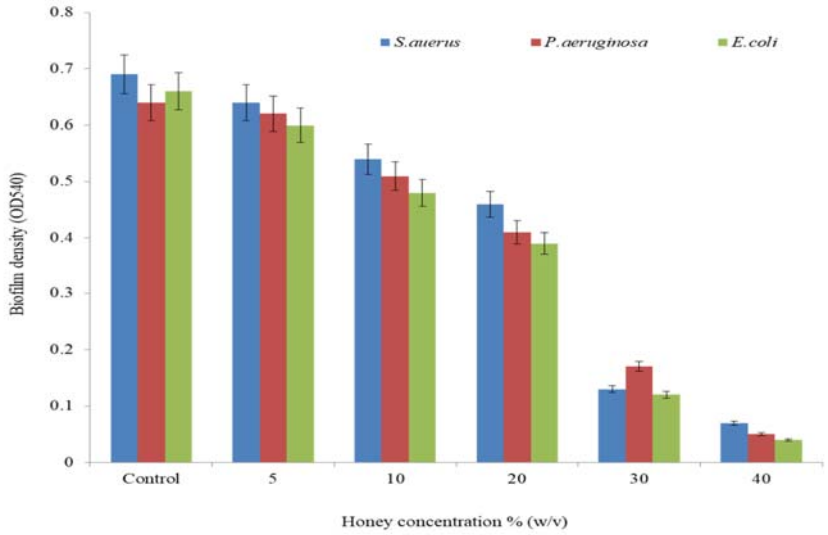

Figure 2: The effect of *Kelulut* honey on biofilm formation. Varying concentrations of honey were incubated with *S.auerus*, *P.aeruginosa* and *E.coli* to determine the lowest concentration required to prevent biofilm formation. The extent of biofilm was assayed by optical density

Determination of biofilm viability (by total cell count)

Treatment of *S.auerus*, *P.aeruginosa* and *E.coli* biofilms established over 48 hours with *Kelulut* honey resulted in up to 4 fold log reductions in TVCs. From biofilm formation in the absence of Kelulut honey, 5.0 x 10^8 c.f.u.m 4.9 x 10^8 and 4.8 x 10^8 of *S.auerus*, *P.aeruginosa* and *E.coli* respectively were recoverable from biofilms. There was an approximately 2.61 log, 2.8 log and 2.78 log reduction in viable cells of *S.auerus*, *P.aeruginosa* and *E.coli* respectively following exposure to 20% (w/v) *Kelulut* honey and 3.1 log, 3.5 log and 3.2 log reduction in viable cells of *S.auerus*, *P.aeruginosa* and *E.coli* respectively after treated with 30% (w/v) *Kelulut* honey compared to untreated biofilm. The reduction in viable cells was even more marked using 40% (w/v) *Kelulut* honey, resulting in a 4.3 log, 4.2 log and 4.4 log reduction of *S.auerus*, *P.aeruginosa* and *E.coli* respectively compared to untreated biofilm (Figure 3).

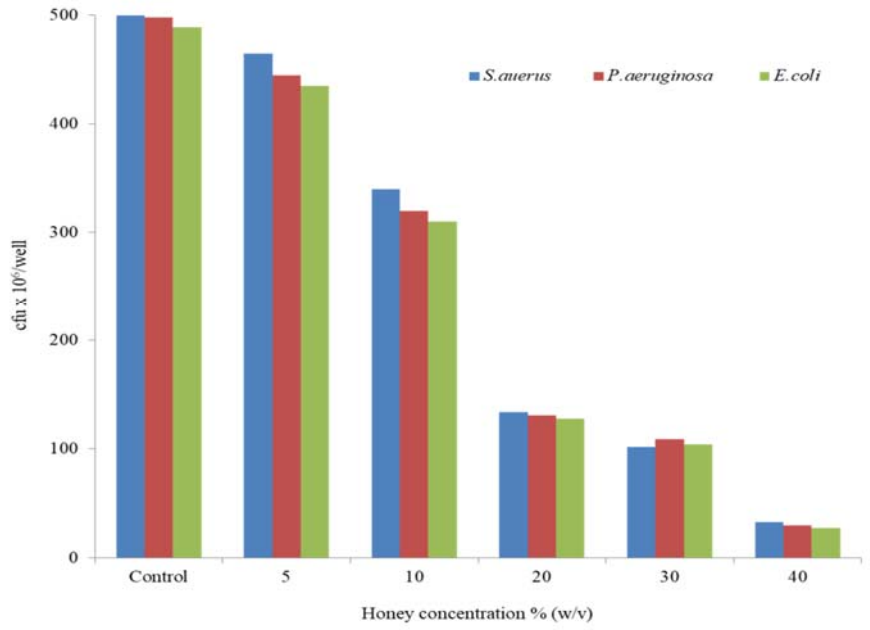

Figure 3: The effect of *Kelulut* honey on biofilm formation of *S.auerus*, *P.aeruginosa* and *E.coli* determined by total cell viability.

Gene expression of *S. aureus* after exposure to *Kelulut* honey

As shown in Figure 4 and Table 4, the expression of seven corresponding genes of *S. aureus* did show the level of different expression (Figure 2). Four genes (*argF*, *purC*, *adh*, and *fabG*) had significantly (P<0.05) decreased levels of expression 1.5-fold, 2.2-fold, 3-fold and 1-fold (P<0.05) respectively, and three genes (*scdA*, *pyk* and *menB*) showed increased expression 5-fold, 7-fold and 3-fold (P<0.05) respectively after honey treatment.

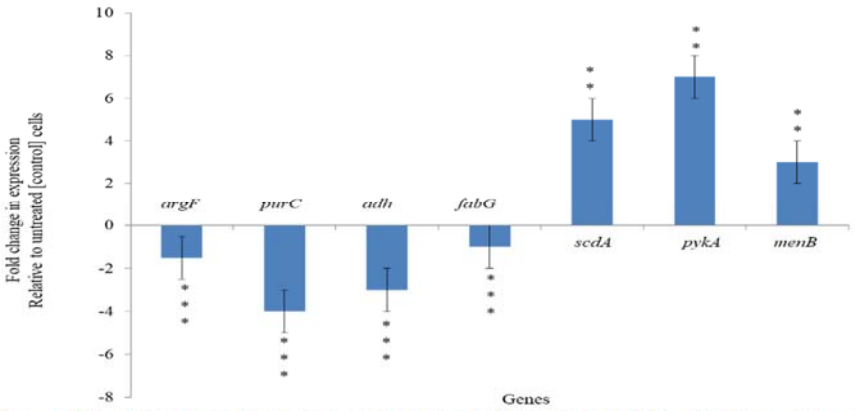

Figure 4: shows the alterations in gene expression profiles associated with the exposure of *S. aureus* to *Kelulut* honey as determined by qPCR. Experiments were run with three technical replicates of each. Mean values of fold changes (± SD) are shown in relation to untreated (control) *S. aureus* cells. Asterisks **$P \leq 0.01$; ***$P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated samples and control.

Table 4: Genes down and up regulated in *S. aureus* detected by RT-qPCR after exposure to *Kelulut* honey

Gene name	Average ΔΔCt	Expression Fold Change (2^-ΔΔCt)	Expression Fold Change	*P*-value	SD
argF	0.59	0.66	-1.5	0.04 *	1.2
purC	2.09	0.23	-4	0.04 *	1.3
adh	1.50	0.35	-3	0.04 *	1.1
fabG	0.01	0.99	-1	0.04 *	1.3
scdA	-2.32	5.00	5	0.04 *	1.0
pykA	-2.81	7.00	7	0.04 *	1.2
menB	-1.58	3.00	3	0.04 *	0.8

Gene expression of *P.aeruginosa* after treatment with *Kelulut* honey

Based on Figure 5 and Table 5, seven genes of *P.aeruginosa* showed different degrees of gene expression including two genes (*fliA* and *fliC*) of flagellum-associated genes that were significantly ($P<0.05$) decreased 3-fold and 4.5-fold respectively. Three genes (*oprB*, *oprH* and *oprC)* associated with the outer membrane were decreased 2-fold and 6-fold respectively except *oprC* which was increased 4-fold, and two genes (*lasR* and *algU*) associated with biofilm formation were increased 2.3-fold and 4-fold respectively in the level of gene expression.

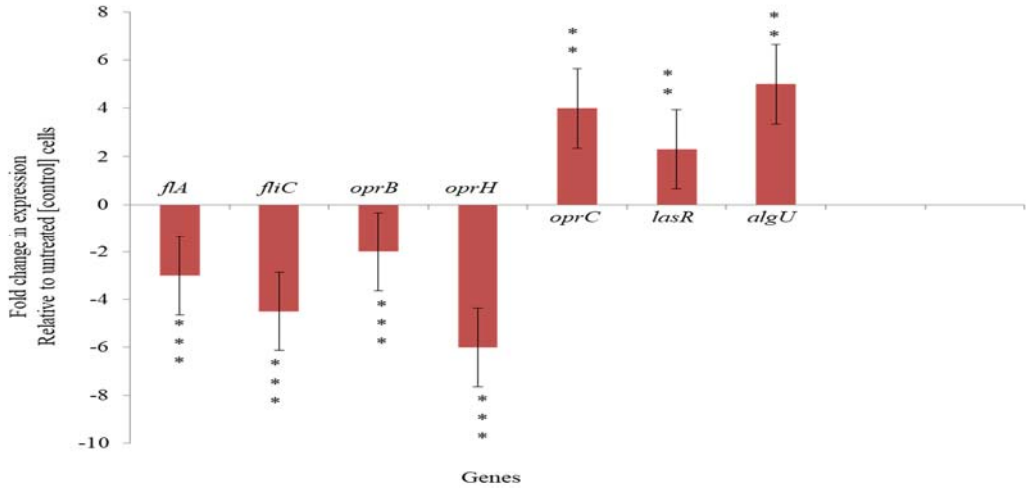

Figure 5: shows the alterations in gene expression profiles associated with the exposure of *P. aeruginosa* to *Kelulut* honey as determined by qPCR. Experiments were run with three technical replicates of each. Mean values of fold changes (± SD) are shown in relation to untreated (control) *P. aeruginosa* cells. Asterisks **$P \leq 0.01$; ***$P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated samples and control.

Table 5 : Genes down and up regulated in *P. aeruginosa* detected by RT-qPCR after being treated with *Kelulut* honey

Gene name	Average ΔΔCt	Expression Fold Change (2^-ΔΔCt)	Expression Fold Change	*P*-value	SD
fliA	1.58	0.33	-3	0.04 *	1.8
fliC	2.17	0.22	-4.5	0.04 *	2.1
oprB	1.00	0.50	-2	0.04 *	1.6
oprH	2.58	0.17	-6	0.04 *	2.3
oprC	-2.00	4.00	4	0.04 *	1.1
lasR	-1.02	2.03	2.3	0.04 *	1.0
algU	-2.32	5.00	5	0.04	1.0

Gene expression of *E.coli* after exposure to *Kelulut* honey

According to Figure 6 and Table 6, six genes (*yjfO (bsmA), ycfR (BhsA), tnaA* and *lsr, rpoS* and *evgA*) involved in biofilm formation, quorum sensing and stress survival in *E.coli* were significantly ($P<0.05$) decreased in the level of gene expression, 5-fold, 3-fold, 8-fold and 2-fold respectively except *rpoS* and *evgA* genes which were increased 7-fold and 4-fold respectively after treatment with honey.

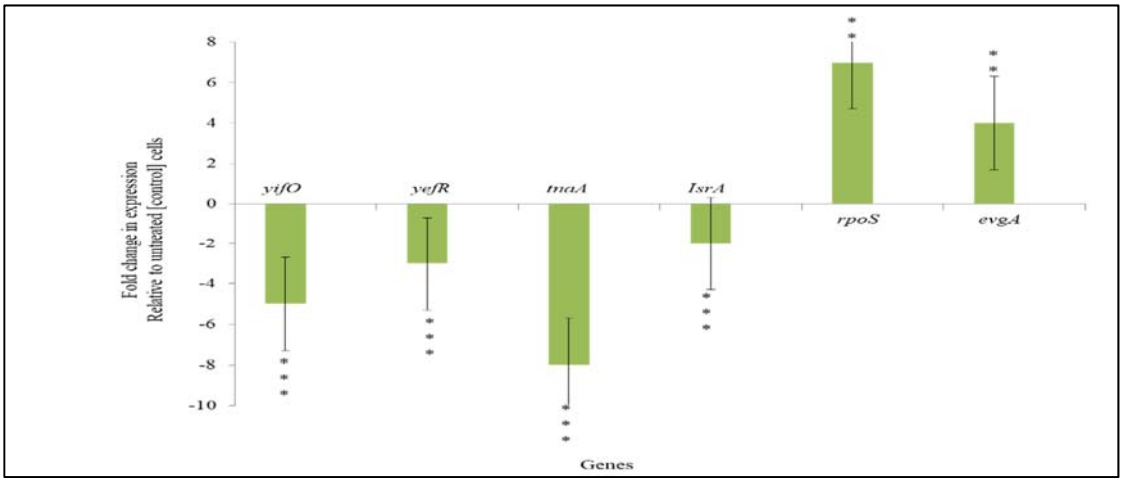

Figure 6: shows the alterations in gene expression profiles associated with the exposure of E. coli **to *Kelulut* honey as determined by qPCR. Experiments were run with three technical replicates of each. Mean values of fold changes (± SD) are shown in relation to untreated (control)** E. coli **cells. Asterisks **$P \leq 0.01$; ***$P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated samples and control.**

Table 6 : Genes down and up regulated in *E. coli* detected by RT-qPCR after treatment with *Kelulut* honey

Gene name	Average ΔΔCt	Expression Fold Change (2^-ΔΔCt)	Expression Fold Change	*P*-value	SD
yjfO (bsmA)	2.32	0.20	-5	0.04 *	2.1
ycfR (BhsA)	1.59	0.33	-3	0.04 *	1.6
tnaA	3.00	0.12	-8	0.04 *	2.3
lsrA	1.00	0.50	-2	0.04 *	1.4
rpoS	-2.81	7.00	7	0.04 *	1.2
evgA	-2.00	4.00	4	0.04 *	0.9

DISCUSSION

In this laboratory study, Kelulut honey was found to prevent the formation of *S.auerus, P.aeruginosa and E.coli* biofilms, as well as inhibiting and disrupting established biofilm. Although determining the density of the bacterial growth in each well yielded information rapidly, the entire contents of the well contributed to turbidity, rather than only the biofilm that was adherent on the walls of each well. Whereas this was appropriate in experiments to estimate the concentration of honey needed to prevent and inhibit a biofilm forming. The results show that different bacterial species has different susceptibility to honey, but the differences in susceptibilities may be found between different strains of the same species. As such, the results obtained for each strain cannot be generalized to the whole bacterial species. Equally, the results demonstrate the ability of honey in preventing and inhibiting the growth of biofilm bacteria, even when the concentrations used are very low. Relevantly in Okhiria et al. (2009), Manuka honey was found to considerably decrease the *P.* aeruginosa biofilm at 40% (w/v) concentration of honey, while 30% (w/v) concentration of honey showed no significant inhibition [75]. In fact, the effectiveness of Manuka honey

against *S. aureus* and *P. aeruginosa* biofilms has been reported [76]. Manuka honey was also found to interrupt the preformed biofilms of *Streptococcus pyogenes* and *Pseudomonas aeruginosa* [39, 61, 70]. Relevantly in Ansari et al. (2013), jujube honey was found to disrupt the pre-formed biofilms of *Candida albicans* [77].

Inhibition of biofilm formation can be explained by the presence of flavonoids, previously reported, which are capable of reducing biofilm synthesis because they can suppress the activity of the autoinducer-2 responsible for cell-to-cell communication [78-80]. This statement can be applied in this study which supports the effectiveness of honey in reducing biofilm biomass. The presence of lysozyme is able to breakdown the established biofilm by digesting the bacteria [81]. The flavonoid pinocembrin which is believed present in honey is a very unique antibacterial factor [82]. Flavonoid pinocembrin is an antioxidant that is able to kill bacteria and thus might contribute to the reduction of biofilm biomass [82].

As demonstrated by RT-qPCR, the expression of *argF*, *purF*, *adh* and *fabG* genes had decreased, while the genes of *scdA*, *pykA* and *menB* were increased in expression of *S. aureus.* Such results show the ability of *Kelulut* honey in impairing the efficacy of ligand binding that is needed for adherence, demonstrating the improved virulence and biofilm formation in *S. aureus.* Meanwhile, the *agrF* locus comprises a quorum-sensing gene cluster that carries five genes namely *agrB*, *agrD*, agrC, *agrA* and *hla*. These genes ease the generation and the discovery of an autoinducing peptide (AIP) in the regulation of the expression of genes coding for factors of virulence [83-84]. The decreased expression of *argF*, *purF*, *adh* and *fabG* genes in *S. aureus* following the *Kelulut* honey treatment might show that the honey restricts the biofilm formation. Also, the levels of *scdA* gene in *S. aureus* were increased, while changes in the expression of *scdA* will impact the cross-linking of peptidoglycan.

The analysis of gene expression found differential expression of *fliC*, *fliA*, *oprB*, *oprC*, *oprH*, *lasR* and *algU* genes of *P.aeruginosa* when *Kelulut* honey was applied, suggesting the impact of *Kelulut* honey on numerous aspects of the flagellar regulon. In turn, the differential suppression of *fliC* and *fliA* occurred. Hence, the repression of flagella-associated genes allows *Kelulut* honey to mediate the de-flagellation of *P.aeruginosa* which leads to decreased motility, adherence and virulence. Also, it is likely that the reduced expression of the two outer membrane proteins (*oprB* and *oprH*) caused the reduced survivability of *P.aeruginosa*. Furthermore, the exposure to *Kelulut* honey appeared to downregulate the genes of *yjfO (bsmA)* and *ycfR (BhsA)* in *E. coli*. Relevantly, *yjfO (bsmA)* and *ycfR (BhsA)* have been characterized as biofilm-promoting genes in *E. coli* [85-86]. It can therefore be stated that *Kelulut* honey can inhibit or disrupt *E. coli* biofilms.

Genes such as *tnaA* and *lsrA* genes have been shown to greatly affect the quorum-sensing network of *E. coli*, [65]. Following the application of *Kelulut* honey in this study, both genes were downregulated leading to the potential supposition that the tested honey may inhibit quorum-sensing, which means that the honey may reduce the virulence of pathogens such as *E. coli*. Meanwhile, following the *Kelulut* honey treatment, the *evgA* and *rpoS* genes in *E. coli* were upregulated and this finding is in line with that of Blair et al. (2009) who reported the upregulation of *rpoS* and *evgA* genes in *E. coli* after being treated with Manuka honey [64]. Similar to Wasfi et al. 2016, the outcomes of this study also show the downregulation of *rpoS* and *evgA* genes in *E. coli* genes following the honey exposure [60]. It is possible that the similarity in expression pattern denotes the similarity in the phytochemical constituents and/or similarity in the antimicrobial mechanisms of the experimented honeys. Previous study showed that the *sof* and *sfbl* were decreased in the expression of *S. pyogenes* after treated with 20% (w/v) concentration of Manuka honey [70]. Study by Roberts et al., (2014) showed that six genes of *P. aeruginosa* including, *fliA, fliC, flhF, fleN, fleQ* and *fleR*

were reduced in gene expression after treated with 12% (w/v) and 24% (w/v) concentration of Manuka honey [61]. Previous study showed that *tnaA* and *yjfO* (*bsmA*) genes were downregulated in expression of *E. coli* in the range from 12.5 to16.2-fold change after treated with 25% (w/v) concentration of Egyptian honey [60]. Study by Roberts et al., (2012) showed that *algD* of *P. aeruginosa* increased 16-fold in the expression whereas *oprF* decreased 10-fold after treated with 12% (w/v) concentration of Manuka honey [39]. Previous study reported that *ycfR* (*BhsA*) and *evgA* genes of *E. coli* were upregulated in expression in the range from 2.2 to 4.19-fold respectively after treated with 25% (w/v) concentration of Egyptian honey [60]. Study by Al-kafaween et al., (2020) showed that the expression of *sof* and s*fbl* decreased 7.82-fold and 9.23-fold respectively, whereas the expression of *algD* and *oprF* decreased 6.28-fold and 11.11-fold respectively after exposure to 20% (w/v) concentration of Kelulut honey [53].

Honey contains various polyphenols, which differs according to the origin and bee species [87, 88]. Various polyphenols, of which some are also detected in honey, have been proven to curb the development of many diseases. They perform this action via several specific mechanisms such as regulation of a specific gene expression or altering metabolic pathways by means of promoting or blocking specific pathways [87, 89]. However, differences in honey samples may affect the type of polyphenols found in honey. As one type of honey might not contain all of the polyphenols and the protective effects of polyphenols are varied, it is advisable to consume variety of honey samples . The therapeutics effects of stingless bee honey such as antidiabetic, wound healing, anticancer, treatment of eye disease, and effects of fertility as proven by many scientific studies [87, 88]. Among the physicochemical parameters of honey, the acidity and the osmolarity represent the principal factors responsible for the antimicrobial activity of honey. However, there are other factors that are closely related to the antimicrobial capacity of honey such as the hydrogen peroxide content, and other non-peroxide components such as methylglyoxal, the antimicrobial peptide bee defensin-1, polyphenols and other compounds from the bees [69]. Related gene expression in honey was reflected as down-regulation of spaetzle, AMPs abaecin and defensin-1 and up-regulation of lysozyme-2 [90].

CONCLUSION

In this study, we compared patterns of gene expression in *E. coli, S. aureus* and *P. aeruginosa* cells treated with and without *Kelulut* honey. We have also shown that *Kelulut* honey was able to reduce biofilm formation of *E. coli, S. aureus* and *P. aeruginosa*. Differential gene expression in response to honey exposure exhibited downregulation of several genes of *S. aureus, P. aeruginosa* and *E. coli*. The obtained results indicate that *Kelulut* honey may represent promising antibiofilm and anti-virulence agent for treatment and modulation of infections caused by *E. coli, S. aureus* and *P. aeruginosa*.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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تأثير عسل الكيلولوت الماليزي على تكوين الأغشية الحيوية والتعبير الجيني للمكورات العنقودية الذهبية الزائفة الزنجارية والإيشريكيا كولاي

محمد الكفاوين[1]،أبو بكر حلمي[1]،حامد الجمال[1]،نورزواني جفار[1]،هبه السيد[2]،محمد زهري[1]

1 كلية علوم الصحة، جامعة السلطان زين العابدين، ماليزيا

2 كلية الصيدلة وعلوم الطب، جامعة البتراء،الأردن

ملخص

يشتهر العسل الآن كعلاج بديل بسبب نشاطه المضاد للبكتيريا واسع الطيف وعدم قدرة البكتيريا على تطوير المقاومة بعد التعرض له. ثبت أن العسل مبيد للجراثيم ضد المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي لأنه يزعزع استقرار جدار الخلية البكتيرية. صممت هذه الدراسة لتقييم تأثير عسل الكيلولوت الماليزي على الأغشية الحيوية ، والغشاء الحيوي الوقائي ، وعلى مستوى التعبير الجيني في المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي. تم إجراء فحوصات الأغشية الحيوية والغشاء الحيوي الوقائي على ثلاث المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي باستخدام لوحة ميكروتيتر مع خمسة تراكيز مختلفة من العسل وهي 5٪ ، 10٪ ، 20٪ ، 30٪ ، 40٪ ٪ (وزن / حجم). ومستوى مقياس التعبير الجيني باستخدام النسخ العكسي الكمي (**RT-qPCR**). كان عسل الكيلولوت قادرًا على تقليل تكوين كتلة الأغشية الحيوية بنسبة تصل إلى 39٪ و 41٪ و 37٪ في المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي على التوالي. أقل تركيز لعسل الكيلولوت وجد أنه يمنع تكون الأغشية الحيوية في المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي كان 30٪ (وزن / حجم). لوحظ انخفاض في العدد الإجمالي للخلايا البكتيرية القابلة للحياة لكل من المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي بعد العلاج بنسبة 40٪ (وزن / حجم) من عسل الكيلولوت .أظهر **RT-qPCR** أن اثني عشر جينًا وتشمل **argF** و **purC** و **adh** و **fabG** و **fliA** و **fliC oprB** و **oprH** و **yjfO (bsmA)** و **ycfR (BhsA)** و **lsrA** و **tnaA** تم انخفاض مستوى التعبير الجيني بعد التعرض لعسل الكيلولوت ، بينما تم ارتفاع مستوى التعبير الجيني لثمانية جينات وتشمل **scdA** و **pyk** و **menB** و **oprC** و **lasR** و **algU** و **rpoS** و **evgA** بعد التعرض لعسل الكيلولوت .أظهرت هذه الدراسة فاعلية عسل الكيلولوت الماليزي فاعليته ضد الأغشية الحيوية ، وأن التراكيز المختلفة من العسل لها درجات مختلفة من التأثير المحتمل على الأغشية الحيوية الراسخة. أيضًا ، سيؤثر انخفاض التعبير عن جينات الفوعة في هذه البكتيريا على قابليتها للأمراض..

الكلمات الدالة: المكورات العنقودية الذهبية، الزائفة الزنجارية والإيشريكيا كولاي، التعبير الجيني ، عسل كيلولوت الماليزي، البيوفيلم.

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Pharmacy students' attitudes to provide rational pharmaceutical care: A multi-institutional study in Jordan

Mohammad B. Nusair[1], Hamza Alhamad[2], Tareq Mukattash[3], Raghad Al-sheyyab[4], Sayer Alazzam[3]

1Department of Clinical Pharmacy and Pharmacy Practice, Faculty of Pharmacy, Yarmouk University, Irbid- Jordan

2 Faculty of Pharmacy, Zarqa University, Zarqa, Jordan

3 Department of Clinical Pharmacy, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid- Jordan

ABSTRACT

This study aims to assess the Jordanian pharmacy students' attitudes and perceptions toward providing rational pharmaceutical care. The study was conducted by administering an anonymous online survey (Standard Pharmaceutical Care Attitude Survey; PCAS) to pharmacy students at five public universities in Jordan. Descriptive statistics were used to describe participants response on the questionnaire items and one-way ANOVA were used for inferential statistics. A total of 884 responses met the eligibility criteria. The majority of participants (n=614, 69.5%) reported that they have been introduced to the pharmaceutical care concept in their program. The majority of participants reported positive attitudes toward the professional duty and benefit of pharmaceutical, more negatives attitudes were reported toward the return on effort. Participants who had a clerkship experience had significantly more positive attitudes toward six items of the PCAS. In conclusion, pharmacy students in Jordan have positive attitudes toward providing rational pharmaceutical care. The majority, however, perceived pharmaceutical care to be time-consuming and not worth the additional workload that it places on pharmacists. This study highlights opportunities to achieve more positive attitudes by mandating pharmaceutical care courses in pharmacy schools in Jordan and exposing students to real-world scenarios.

Keywords: Pharmacy education; Pharmaceutical care; Pharmacy students; Jordan

1. INTRODUCTION

Advances in pharmacy practice and the introduction of pharmaceutical care have transformed the pharmacy profession from traditional dispensing practices to more patient-centred care.[1] Pharmaceutical care has an impact on enhancing patient's medications and improving patients' quality of life.[2] Pharmacists are currently providing more services to patients in addition to the traditional role of dispensing medication.[1] As an integral part of the health care team, the pharmacist takes responsibility for a patient's medication-related needs and problems, optimizing patient's use of medicine, reducing unnecessary medicine and polypharmacy, and improving patient's quality of life.[3]

The advancement of the pharmacist's role to be part of the health care team to maximize the health care provided to the patient should be met with expanding the scope of the pharmacist from being a medicine compounder, dispenser, or seller to include pharmaceutical care provision.[4]

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Pharmaceutical care is an essential element of healthcare that involves patient interviewing to take medication history, identifying, preventing, and resolving potential and actual medication-related problems such as drug-drug interaction, adverse drug reactions, polypharmacy, unnecessary medications, and designing an evidence-based care plan.[2,5] A recent study from Jordan reported that the lack of patient-centred practices and medication review services while providing pharmaceutical care might contribute to the increase in the drug-drug interactions and drug-related problems.[6]

Pharmacy profession should be redirected in education and practice toward pharmaceutical care services by changing the undergraduate pharmacy curriculum to be more oriented to pharmaceutical care services.[7,8] Also, new methods to enhance students' ability to understand new skills concerning pharmaceutical care practice, such as adding clinical and patient-oriented courses that help in identification, analysis, and prevention of drug therapy-related problems, should be a primacy for all pharmacy schools in Jordan.[7,8] To update the curricula of pharmacy education in Jordan, pharmacy schools are required to update their curricula[7] to mimic the changes in the pharmacy profession that have taken place in more developed countries.[1]

The BSc. in pharmacy curricula in Jordan has little focus on pharmaceutical care skills, with little patient-oriented programs training courses.[8,9] For instance, only Jordan University of Science and Technology and the University of Jordan have a mandatory pharmaceutical care course in their BSc. pharmacy and PharmD curricula.[10-12] Other universities either having an elective pharmaceutical care course such as Yarmouk university[13] or teaching pharmaceutical care in one course integrated with communication skills such as the Hashemite University.[14] While in other universities' pharmaceutical care, we could not identify pharmaceutical care in their curricula (e.g., Mutah University).[15] However, an ongoing plan of curricula changes based on the Center for the Advancement of Pharmacy Education (CAPE) outcomes has been reviewed and approved by all pharmacy school deans in Jordan. These new curricula will be implemented within the next two years and should incorporate more pharmaceutical care credit hours.

Pharmacy students' attitudes and perceived preparedness can be used as an influence and assessment tool to conduct pharmacy curricular change, revise, and improve pharmacy curricula.[16,17] Regrettably, the concept of pharmaceutical care among Jordanian pharmacy students, has not been studied yet. Therefore, this study aims to assess the Jordanian pharmacy students' attitudes and perceptions toward providing rational pharmaceutical care.

Methods

Design

This cross-sectional study aimed to describe Jordanian pharmacy students' attitudes toward pharmaceutical care. The study was conducted at the beginning of the summer semester between July and August 2019. An anonymous online survey (Standard Pharmaceutical Care Attitude Survey; PCAS) to pharmacy students at public universities in Jordan. The study's protocol was approved by the Institutional Review Board (IRB) at King Abdullah University Hospital (reference number: 76/117/2018).

Participants

The study protocol aimed to recruit pharmacy students from all pharmacy schools in Jordan. However, the researchers had limited access and responses from private universities' students. Therefore, we modified our study protocol to invite pharmacy students in public universities to take part in this study. There are five public universities in Jordan that have pharmacy and PharmD programs: the university of Jordan (BSc. pharmacy and PharmD), Yarmouk University (BSc. Pharmacy), Jordan University of Science and Technology (BSc. pharmacy and PharmD), Mutah University (BSc. Pharmacy) and the Hashemite University (BSc. Pharmacy). Pharmacy and PharmD students from these public universities were invited to participate in this study

through social media groups (e.g., Facebook). The study protocol aimed to target students who were in their 3rd,4th,5th, and 6th academic year since first and second year courses are primarily introductory and basic science courses.[10-15] Therefore, responses from 1st and 2nd-year students were excluded. Participants were provided with an electronic information study sheet and provided an online form of informed consent before taking the survey.

PCAS Survey

The online survey was prefaced with pharmaceutical care definition according to Helper and Strand [18] and consisted of two sections. The first part consisted of eight questions to elicit demographic information of participants. The second part was the 13-item PCAS that aimed to assess participants' attitudes toward pharmaceutical care. PCAS is a five-point Likert scale (1=strongly disagree to 5=strongly agree) with evidence of validity and reliability and has been extensively used in the literature.[19-23] PCAS measures three constructs: professional duty (three questions), return on effort (two questions), and professional benefit (eight questions). Return on effort items were negatively worded and reverse scored so that the higher scores represent more positive attitudes toward pharmaceutical care. The survey questions were all written in English with an Arabic translation for each question to ensure participants understanding for all questions. The survey was piloted on 19 pharmacy students for face validity and no modifications were suggested. The pilot sample completed the survey in 4.5 minutes on average and their responses were not included in the final results.

Data Collection and Analysis

Data were collected using SurveyMonkey® (SurveyMonkey, Palo Alto, CA, USA) and exported directly to Statistical Package for Social Sciences (SPSS) software version 25 for analysis. Descriptive statistics used to analyze responses (i.e., mean score with standard deviations and percentage frequencies). In order to facilitate interpretation across the three constructs of PCAS, summated total scales were transformed to a 0-100 scale. Cronbach's alpha was calculated to estimate the internal consistency of the responses to PCAS. One-way ANOVA was used to assess the influence of socio-demographic characteristics of participants on their attitudes toward pharmaceutical care.

Results

A total of 1098 responses were completed, and 214 responses were excluded as they came from first- and second-year students. The response rate could not be calculated based on the Facebook groups members as many of these members did not meet the inclusion/exclusion criteria. However, we estimated the response rate to be 19.6% based on the total number of students enrolled in the eligible academic years at all five public universities. Out of the 884 responses who met the eligibility criteria, 700 (79.2%) were females with an average age of 22.04 years (Table 1). The majority of participants (n= 605, 68.4%) were enrolled in a BSc pharmacy program 605 (68.4%) and have had an internship in a community pharmacy and/or hospital setting (n=673, 76.1%). The majority of participants (n=614, 69.5%) reported that they have been introduced to the pharmaceutical care concept in their program (Table 1). The Cronbach alpha was found to be 0.746 for the 13 PCAS items, which indicate acceptable overall reliability.

Table 1: Sociodemographic characteristics of participants (n=884)

Variable		N(%)
Gender	Female	700 (79.2%)
	Male	184 (20.8%)
Academic Year	Third	203 (23%)
	Fourth	299 (33.8%)
	Fifth	327 (37%)
	Sixth	55 (6.2%)

Variable		N(%)
University	University of Jordan	249 (28.2%)
	Yarmouk University	210 (23.8%)
	Jordan University of Science and Technology	217 (24.5%)
	Mutah University	106 (12%)
	Hashemite University	102 (11.5%)
Program	BSc Pharmacy	605 (68.4%)
	PharmD	279 (31.6%)
Cumulative GPA	Excellent	156 (17.6%)
	Very Good	354 (40%)
	Good	306 (34.6%)
	Satisfactory	68 (7.7%)
Took pharmaceutical care course	Yes	614 (69.5%)
	No	270 (30.5%)
Training experience	Training in community pharmacy and/or hospital setting	673 (76.1%)
	No previous training experience	211 (23.9%)
Age (mean ± SD)	22.04 ±1.40	

Overall, participants had positive attitudes toward pharmaceutical care practice (Table 2). The majority of participants reported to have positive attitudes toward the professional duty and benefit of pharmaceutical, more negatives attitudes were reported toward the return on effort (Table 3). Over 90% of participants agreed that pharmacists' primary responsibility to prevent and solve medication-related problems and should perform pharmaceutical care (Table 2). On the other hand, 76.1% of participants agreed that pharmacy students can perform pharmaceutical care during their clerkship. Despite that over half of the participants believed that pharmaceutical care does not take too much time and effort, 58.5% of them reported that it is not worth the additional workload it places on the pharmacists (Table 2).

Table 2: Students' attitudes towards pharmaceutical care per PCAS item (n=884)

		Mean ± SD	Strongly Agree	Agree	Neutral	Disagree	Strongly Disagree
Professional duty	All pharmacists should perform pharmaceutical care	4.56 ± 0.59	527 (59.6%)	331 (37.4%)	19 (2.1%)	6 (0.7%)	1 (0.1%)
	The primary responsibility of pharmacist in health care settings should be to prevent and solve medication problems	4.34 ± 0.71	396 (44.8%)	415 (46.9%)	53 (6.0%)	3 (0.3%)	0
	Pharmacists primary responsibility should be to practice pharmaceutical care	4.20 ± 0.77	337 (38.1%)	410 (46.4%)	108 (12.2)	29 (3.3%)	0
Return on effort	Providing pharmaceutical care takes too much time and effort*	1.59 ± 1.22	63 (7.1%)	185 (20.9%)	140 (15.8%)	319 (36.1%)	177 (20%)

		Mean ± SD	Strongly Agree	Agree	Neutral	Disagree	Strongly Disagree
	Providing pharmaceutical care is not worth the additional workload that it places on the pharmacist*	2.44 ± 1.25	185 (20.9%)	332 (37.6%)	140 (15.8%)	142 (16.1%)	85 (9.6%)
Professional benefit	Pharmacy students can perform their pharmaceutical care during their clerkship	3.97 ± 0.90	268 (30.3%)	405 (45.8%)	135 (15.3%)	74 (8.4%)	2 (0.2%)
	I think the practice of pharmaceutical care is valuable	4.53 ± 0.56	494 (55.9%)	369 (41.7%)	17 (1.9%)	4 (0.5%)	0
	I would like to perform pharmaceutical care as a pharmacist practitioner	4.45 ± 0.64	459 (51.9%)	378 (42.8%)	36 (4.1%)	10 (1.1%)	1 (0.1%)
	Providing pharmaceutical care is professionally rewarding	4.08 ± 0.89	318 (36%)	388 (43.9%)	119 (13.5%)	51 (5.8%)	8 (0.9%)
	I feel that the pharmaceutical care is the right direction for the provision to be headed	4.41 ± 0.67	438 (49.5%)	379 (42.9%)	55 (6.2%)	12 (1.4%)	0
	I feel that pharmaceutical care movement would benefit pharmacists	4.40 ± 0.69	437 (49.4%)	377 (42.6%)	53 (6%)	16 (1.8%)	1 (0.1%)
	I feel that pharmaceutical care movement will improve patient health	4.56 ± 0.56	525 (59.4%)	335 (37.9%)	21 (2.4%)	3 (0.3%)	0
	I feel that practicing pharmaceutical care will benefit my professional pharmacy career as a pharmacy practitioner	4.51 ± 0.63	507 (57.4%)	333 (37.7)	34 (3.8%)	9 (1%)	1 (0.1%)

* items were reverse scored.

Table 3: PCAS constructs descriptive analysis (scores transformed to a 0-100 scale)

	Mean ± standard deviation	Minimum: Maximum score	Range
Professional Duty	87.27 ± 10.39	40:100	60
Return on Effort	60.32 ± 19.36	20:100	80
Professional Benefit	87.29 ± 9.29	45:100	55

Students' attitudes toward pharmaceutical care were influenced by some socio-demographic variables (Table 4). Females participants had significantly more positive attitudes toward the two times of return on effort among three other items (Table 4). BSc pharmacy students had significantly had more positive attitudes toward the value of pharmaceutical care than PharmD students. Moreover, participants who had an internship in community

pharmacy and/or hospital settings had significantly more positive attitudes toward the clerkship item among five other items when compared to participants who did not have any internship (Table 4).

Table 4: Statistically significant association between sociodemographic characteristics and PCAS items

Demographic variable	PCAS question	Demographic variable subcategories		P value
Gender		Male	Female	
	All pharmacists should perform pharmaceutical care	4.45 ± 0.57	4.59 ± 0.59	.004
	Providing pharmaceutical care takes too much time and effort	2.05 ± 1.30	1.47 ± 1.17	< .001
	Providing pharmaceutical care is not worth the additional workload that it places on the pharmacist	2.84 ± 1.7	2.66 ± 1.25	< .001
	I think the practice of pharmaceutical care is valuable	4.40 ± 0.56	4.57 ± 0.56	< .001
	I feel that pharmaceutical care movement will improve patient health	4.48 ± 0.58	4.58 ± 0.55	0.030
Program		BSc Pharmacy	PharmD	
	I think the practice of pharmaceutical care is valuable	4.57 ± 0.55	4.46 ± 0.57	.041
Training		No previous internship	Had an internship	
	All pharmacists should perform pharmaceutical care	4.47 ± 0.64	4.58 ± 0.57	.017
	Pharmacists primary responsibility should be to practice pharmaceutical care	4.03 ± 0.75	4.24 ± 0.77	.001
	Providing pharmaceutical care is not worth the additional workload that it places on the pharmacist	2.36 ± 1.18	2.47 ± 1.27	.041
	Pharmacy students can perform their pharmaceutical care during their clerkship	3.81 ± 0.82	4.02 ± 0.92	.002
	I feel that the pharmaceutical care is the right direction for the provision to be headed	4.29 ± 0.71	4.44 ± 0.65	.004
	I feel that pharmaceutical care movement will improve patient health	4.48 ± 0.58	4.59 ± 0.55	.018
	I feel that practicing pharmaceutical care will benefit my professional pharmacy career as a pharmacy practitioner	4.39 ± 0.70	4.55 ± 0.62	.001
Haven been introduced to pharmaceutical care at school		Yes	No	
	All pharmacists should perform pharmaceutical care	4.59 ± 0.57	4.49 ± 0.61	.021
	I feel that pharmaceutical care movement would benefit pharmacists	4.43 ± 0.68	4.31 ± 0.711	.023
	I feel that pharmaceutical care movement will improve patient health	4.60 ± 0.55	4.48 ± 0.58	.004
	I feel that practicing pharmaceutical care will benefit my professional pharmacy career as a pharmacy practitioner	4.54 ± 0.62	4.44 ± 0.66	.037

- One-way ANOVA test was used. Statistical significance represented by $p < 0.05$

Discussion

The Jordanian government has a robust obligation concerning health and education programs. Pharmacy practice and education are developing, evidenced by the increase in the number of pharmacy schools and pharmacy students.[24] To illustrate more, there were eight pharmacy schools before 2009,[25] with eighteen pharmacy schools now.[24] However, the focus of pharmaceutical care in the pharmacy curricula is inefficient and lacking patient-oriented program training.[7,8,26]

Continuous revisions of the pharmacy curriculum to keep up with the changing of the pharmacists' role is needed.[17] Pharmacy students' attitudes and perceived readiness can be used to revise, improve, and influence the pharmacy curricular change. This study described the attitudes of Jordanian pharmacy students toward pharmaceutical care. Overall, pharmacy students in this study had positive attitudes toward pharmaceutical care practice. The finding of our study is consistent with the results of many studies where American,[20,21] Nigerian,[27] Nepal,[19] Saudi,[22] Qatari[23] pharmacy students all positive attitudes toward pharmaceutical care practice. However, pharmacy students in this study had negative attitudes toward the return on effort for providing pharmaceutical practice. This is somehow consistent with previous studies in the Middle East region.[22,23]

Data from our study suggested that some socio-demographic variables influenced students' attitudes toward pharmaceutical care. Females found to have more positive attitudes towards pharmaceutical care than males. Findings were supported by the results of previous studies.[22,27] Moreover, participants who have been introduced to pharmaceutical care in their program or had a clerkship experience had significantly higher positive attitudes when compared to those who did not. Similarly, Chisholm and Wade reported that an overall increase in students' attitudes toward pharmaceutical care when they were introduced to actual patients in the classroom.[28] This concordance suggests that we can achieve higher students' attitudes toward pharmaceutical care when training our students with real-world scenarios in the classroom and in clerkship.

The scope of practicing pharmaceutical care, attitudes and barriers toward this practice have not been thoroughly examined in Jordan,[9] despite the importance of implementing pharmaceutical patient care in ensuring medication safety, enhancing cost-effective use of medicines, reducing medicines related morbidity and mortality and its economic burden.[3,29,30] Although Jordanian pharmacists have positive attitudes toward the implementation of pharmaceutical care practice, several barriers have been identified which limit the implementation of pharmaceutical care practice in Jordan.[7,9] Those barriers include the level of knowledge about providing pharmaceutical care, lack of a private counseling area at the community pharmacies, communicational difficulties in with physicians, lack of access to patient medical records,[9] and weak emphasis on pharmaceutical care education and training of the undergraduate pharmacy education (i.e., BSc. in Pharmacy and PharmD) curricula in Jordan.[8,26]

The slow change in the education programs and lack of pharmaceutical care jobs contribute to the present situation where the primary roles of the pharmacist in Jordan are more dispensing and marketing.[8] In contrast, the future of pharmaceutical care in Jordan looks bright with the establishment of PharmD and Master of clinical pharmacy programs at the major universities in Jordan since the last two decades. Consequently, the role of the pharmacist in Jordan is expected to be expanded as a drug therapy consultant while providing pharmaceutical care, which is considered possible to be applied in Jordan.[8]

Strengths and Limitations

The strength of our study is that it is the first study conducted in Jordan to assess the pharmacy students' attitude, perception toward providing pharmaceutical care using the PCAS assessment tool. However, our study has limitations that need to be taken into consideration while

interrupting the results. Data from our study are self-reports of student's attitudes toward pharmaceutical care, where there is a risk that students may describe themselves in a more favorable light or to appear or referred to as hard-working students (i.e., social desirability bias). Moreover, the results of positive pharmacy students' attitudes towards providing pharmaceutical care from our study in Jordan may not be generalizable to other private schools of pharmacy in Jordan.

Our study is the first to evaluate pharmacy students' attitudes toward providing pharmaceutical care and is considered a vital contribution that helps influence the pharmacy curricula revision and change. Our study provides a piece of evidence to explore changes regarding the shift toward pharmaceutical care education and practice in Jordan. Results from our study are expected to influence the policy decision-making process of updating the pharmacy school curricula taking into consideration the changes in the pharmacy profession that have taken place in more developed countries. This is significant and can influence the ongoing plan of curricula changes that have reviewed and approved by pharmacy schools' deans based on the CAPE educational outcomes. These changes mandate more pharmaceutical care credit hours be implemented within the next two years.

Conclusion

Overall, pharmacy students in Jordan have positive attitudes toward providing rational pharmaceutical care. The majority, however, perceived pharmaceutical care to be time-consuming and not worth the additional workload that it places on pharmacists. This study highlights opportunities to achieve more positive attitudes by mandating pharmaceutical care courses in pharmacy schools in Jordan and exposing students to real-world scenarios.

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توجهات طلبة الصيدلة نحو تقديم الرعاية الصيدلانية المُثلى: دراسة متعددة المؤسسات في الاردن

محمد نصير[1]، حمزه الأحمد[2]، طارق مقطش[3]، رغد الشياب،ساير العزام[3]

1الصيدلة السريرية والممارسة الصيدلانية،كلية الصيدلة ،جامعة اليرموك،الأردن
2كلية الصيدلة ،جامعة الزرقاء ،الأردن
3الصيدلة السريرية والممارسة الصيدلانية،كلية الصيدلة،كلية العلوم والتكنولوجيا،الأردن

ملخص

تهدف هذه الدراسة إلى تقييم اتجاهات وتصورات طلبة الصيدلة في الأردن حول تقديم الرعاية الصيدلانية وذلك من خلال استبيان محكمّ تم توزيعه عبر الإنترنت. استهدفت هذه الدراسة طلبة الصيدلة في خمس جامعات حكومية في الأردن من خلال مجموعات التواصل الاجتماعي الخاصة بطلبة الكليات الخمسة. أكمل تعبئة الاستبيان ما مجموعه 884 طالب، وأفاد غالبية المشاركين (ن = 614 ، 69.5٪) أنهم قد تعرّفوا على مفهوم الرعاية الصيدلانية خلال دراستهم. كما وجدت الدراسة أنَّ غالبية المشاركين كانت لديهم مواقف إيجابية تجاه الواجب المهني وفوائد الرعاية الصيدلانية. ومع ذلك، اعتبرت الغالبية أن الرعاية الصيدلانية تستغرق وقتًا طويلاً ولا تستحق عبء العمل الإضافي الذي تضعه على كاهل الصيادلة. كما وجدت الدراسة أنَّ الطلبة الذين لديهم تجربة في التدريب الميداني كان لديهم مواقف أكثر إيجابية تجاه ستة عناصر مقارنة بالطلبة الذين لم يكن لديهم تجربة في التدريب الميداني. تسلّط هذه الدراسة الضوء على فرص تحقيق مواقف أكثر إيجابية وذلك بأن تكون مادة الرعاية الصيدلانيه مادة اجباريه في كليات الصيدلة في الأردن وتقديم حالات عملية تحاكي الواقع لإعداد الطلبة ودفعهم للانخراط بسوق العمل وتقديم الرعاية الصيدلانية المثلى للمرضى..

الكلمات الدالة: التعليم الصيدلاني؛ الرعاية الصيدلانية؛ طلبة الصيدلة؛ الأردن.

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Chemical composition of essential oil from *Semenovia suffruticosa* and their antimicrobial's effects in drinking water

***Alireza.Sardashti* [*1] *and Ali·Kordi Tamandani*[1]**

[1]*Department of Chemistry, Faculty of science, University of Sistan and Baluchestan, P.O.Box 98135-167, Zahedan, Iran.*

ABSTRACT

This study reports the chemical composition and antibacterial activity of the essential oil from aerial parts *of Semenovia suffruticosa* (which is endemic to Iran). The essential oil of aerial parts extracted by hydro distillation method and was investigated using GC& GC-MS techniques. The oil yield (w/w %) was 0.77 on dry weight. Sixty components were recognized consists of 84.99 % of total oil .The major components found in the oil: cis-ocimene (19.31%), linalool (9.00), cinnamy valerate(8.19%), α-terpinolene (5.49%) ,6-amyl-α-pyrone(4.55%) and unknown compounds (1.70 %).The percentage of oxygenated terpenoids and total terpenoids in the essential oil are 22.69 and 61.42 respectively. Antimicrobial effects of this oil were examined according to Agar dilution method. The results of Minimal inhibition Concentration presented, in the following: *Staphylococcus aureu*s (8 $\mu g.mL^{-1}$), *Salmonella typhi* (32 $\mu g.mL^{-1}$), *Escherichia coli* (32 $\mu g.mL^{-1}$), *Candida albicans*(8 $\mu g.mL^{-1}$), *Aspergillus niger*(32 $\mu g.mL^{-1}$), shows that the highest dilution of essential oil has been on *Candida albican*s and the lowest dilution of essential oil that is capable of control on micro-organism, has been *pseudomonas aeruginosa* . The antimicrobial effects of essential oil have been demonstrated in drinking water using the heterotrophic plat technique. The increase of this essential oil among the samples resulted in decreasing the number of micro-organisms colonies.

Keywords: *Umbellifera* , Essential oil composition , Agar dilution method Heterotrophic plat count (H.P.C) technique.

1. INTRODUCTION

Eleven species of genus *Semenovia* are found in Iran, five of them are endemic[1]. *Semenovia suffruticosa* is a perennial plant species of *Apiaceae* (*Umbeliferae*) family that grows only in altitudes of 2300 - 2500m Taftan Mountain (Sistan & Baluchestan province, Iran). The plant *Semenovia suffruticosa* has comb-like leaves or bifurcate divisions and long petioles with hard pods. The stems are almost cylindrical trunk and 45 – 70 cm long, with low bifurcate branches, and sometimes with shallow grooves on the surface. Terminal Umbels are within 7 – 10 cm radiuses with almost equal parts and glabrous, and 40 - 50 cm long in fruit-containing state *Umbellules* have 12 - 15 flowers and peduncle is shorter thanthe ripe fruit *Apiaceae* family[2] plants contain compounds such as coumarin, furanocoumarin, cromenocoumarin, terpene, sesquiterpene, triterpenoid saponins and acetylene compounds[3].

The essential oils of *Semenovia suffruticosa* (Freyn Bornm) Manden. And *S.Tragioides* Boiss. Manden were extracted from the aerial parts by hydro distillation method .The subject of our previous has been studies, the major components were linalool (13.9%), lavandulyl acetate (11.5 %), (E)-β-ocimene (9.7%) and cinamy isovalerate (9.4%) in the latter[4].The antimicrobial tests were carried out at the department of biological sciences, north Tehran branch, and LA. University of Tehran using the following microorganisms: *Staphylococcus aureus*

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PTCC1113,*Staphylococcus epidermidis*PTCC1349, *Staphylococcus saprophytic* us PTCC 1379 (Gram-positive bacteria), *Salmonella typ*hi PTCC 1185, Shigella Flexnery PTCC 1234 and *Escherichia coli* PTCC 1330(Gram-negative bacteria) identified by Iranian research organization for sciences of technology (IROST)[5] . Bacteria, molds and yeasts that require organic carbon to grow are called heterotrophic. Most bacteria, including many of the bacteria associated with drinking water systems, are heterotrophic. Concentrations of heterotrophic bacteria are determined using a variety of commonly recognized international methods[6].NBH methods use colony formation in culture media to approximate concentrations of heterotrophic organisms in drinking water samples, where bacteria are more common than molds and yeasts. NBH methods do not provide an indication of the specific heterotrophic bacteria present or their sources[7].Heterotrophic bacteria are present in all types of water. In groundwater, concentrations of heterotrophic bacteria are generally low and stable over time. In surface waters and underground water under the direct influence of surface water, their concentrations vary and can be minimized through effective treatment. Treatment of drinking water does not eliminate or inactivate all heterotrophic organisms[8].As a result, these organisms pass through the treatment system to the distribution or plumbing systems. Heterotrophic bacteria can also enter the distribution system through open treated water tanks, during pipe repairs, or due to back-ups of pipeline projects or the addition of new piping systems[8,9] .The National Primary Drinking Water Regulations regulations established by the Environmental Protection Agency of the United States (US EPA) states that the concentration of heterotrophic bacteria is not necessarily an indicator of health effects, but a low concentration of bacteria Heterotrophs in drinking water is associated with good maintenance of the treatment system and distribution network. This regulation establishes that treatment techniques for surface and ground water subject to the influence of surface waters should be used to limit the concentration of heterotrophic bacteria in drinking water to less than 500 $CFU.mL^{-1}$, Measured using the standard method on agar incubated at 35 °C for 48 h[10] .This standard is not based on health considerations, but reflects the fact that at concentrations greater than 500 $CFU.mL^{-1}$, heterotrophic bacteria can interfere with certain methods of recovery of total coliforms and *E. coli* [11,12] .The Drinking Water Inspectorate of England and Wales, based on the Directive of the EU Council on the quality of water intended for human consumption[13], has not established limit Quantitative analysis of the concentrations of heterotrophic bacteria in drinking water, but stated that no abnormal changes in these concentrations should be observed in tap water, treatment plants or service tanks[14]. Measuring the heterotrophic plate count (H.P.C) is an analytical method that can be a useful operational tool for monitoring general bacteriological water quality through the treatment the process and in the distribution system. Each drinking water system will have a baseline range of H.P.C bacteria levels depending on the site-specific characteristics[15].

Image of *Semenovia suffruticosa* plant in Taftan Mountain (April month)

Materials and Methods

Plant material

The aerial parts of *Semenovia suffruticosa* (which is endemic to Baluchestan- Iran) were collected during the flowering stage from the heights of Taftan Mountain in Baluchestan (South eastern of Iran) in June 2010. Plant identification was carried out by Dr. Mozaffarian [16] Botanist in the Research Institute of Forests and Rangelands (Tehran-Iran).

Preparation of sample

The aerial parts were freeze-dried in the shade at the ambient temperature and stored in double-layer paper bags at the room temperature, protected from the direct light, until further analysis[17,18]. They were then sieved to particles with 0.5 mm sizes. All reagents used were of the analytical grade with the highest purity available.

Essential oil isolation

The essential oil was extracted by mixing e g.50 ±0.01g of plant powder with 400±0.1mL in 2 L- balloon of distilled water at 95 ^{0}C temperature for 2.5 h using a Clevenger-type apparatus based on the recirculation of water according to the method recommended in the European Pharmacopoeia[19] .The oil dried over anhydrous sodium sulfate .After filtration, the solvent was removed by distillation under reduced pressure in a rotary evaporator at 30 ^{0}C and the pure oil kept at 4 ^{0}C in the dark until the moment of analysis. After determining the optimum conditions such as water volume, kind of balloon, time of the process of essential oil tracting proper amount of the plant sample[20].

GC-MS Analysis

The analysis of the essential oil was performed using a Hewlett-Packard 6890 Network GC System, equipped with a 60m* 0.25mm id, 0.25µm an HP-5Ms capillary column, and a HP 5973 mass selective detector. Helium was the carrier gas at 1 ml.min^{-1}, the temperature was at 250 ^{0}C and 260 ^{0}C respectively. The column temperature was set at 40 ^{0}C for 1 min, then programmed from 40 ^{0}C to 250 ^{0}C at a rate of 3 ^{0}C.min^{-1}, and finally, held isothermally for 20 min for GC-MS detection an Electron Ionization

System was used with ionization energy of 70 eV.Retention indices were calculated by using the injector and MS transfer line retention times of C_8-C_{26} n- alkanes that were injected with the oil at the same chromatographic conditions according to Van Den Dool method[21].The individual constituents were identified by their identical retention indices, referring to known compounds from the literature and also by comparing their mass spectra with either at the compounds or with the Wiley7 mass spectral database[22].

Antimicrobial activity

The antibacterial, anti-yeast, and antifungal activities were observed by mean of Ager Dilution method within a concentration range of 0.5 -64 ($\mu g.mL^{-1}$). The Minimum Inhibitory Concentration (MIC) of the aerial parts oil was determined for four different bacteria.

Ager Dilution method

Firstly, the mentioned micro-organisms were cultured on culture environment of Muller- Hinton (for bacteria) and sabred Dextrose Ager for fungi in order to obtain a fresh culture to fresh or to prepare) after 24 h at 37 ^{0}C for bacteria and 48h at and sabred Dextrose Ager for fungi in order to obtain a fresh culture to fresh or to prepare after 24 h at 37 ^{0}C for bacteria and 48 h 5 ^{0}C for fungi. The different concentrations given of essential oil were prepared in Ager-having culture environment as a form of two fold 1/2,1/2 ,so that the dilutions were prepared from the concentration of 256 $\mu g.mL^{-1}$ (256,128,64,32,16,8,4,2,1,1/2) according to standard of NCCLS(or CLSI) bacterium should be added 10^4 $CFU.mL^{-1}$. To achieve this at the first a suspension of half Mc. Farland was provided from bacteria, and it was diluted 10 times and then from each bacterium, 5 µg was taken. Thereafter, they were put on the water plots of the Ager culture environment and the different concentration of essential oils. To examine growth and not-growth of micro-organisms, all the plots were kept at certain the temperature. After 24 h for bacteria and 48 h for fungi, the results were analyzed. All experiments were repeated for at least 3 times[23].

Heterotrophic plat count

The Heterotrophic plat count (H.P.C), represents an indication of aerobic and anaerobic bacteria that derive their energy and carbon from organic compounds. The number of these bacteria depends on the composite of culture environment, ancubation period (1-7 d) and the temperature of incubation (20-35) ^{0}C. This group consists of *pseudomonas aeruginosa, Stahylococcus aureus, Escherichia coli, Aspergillus niger* microorganisms. H.P.C bacteria in drinking water is variable and mostly under the influence of the temperature, remained chlorine existence and the concentration of absorbable organic materials. The amount of H.P.C should not exceed 5oo organisms/ml. The H.P.C is a useful parameter to evaluate water quality in distribution systems and water filtering house[24].Utilization of antimicrobial effects (H.P.C technique) of this essential oil was tested for samples of city-sewage (water and waste water). The effective essential oil concentration for its anti-microbial property has been tested in the H.P.C test with 0.1, 0.3, 0.5, and 0.7, 1.1(mL) concentration for *Semenovia suffruticosa* oil (figure 1). All experiments were repeated for at least 3 times.

Statistical analysis

The measurements was done in triplicates to test the reproducibility of them. All results are presented as mean ±S.E. Statistical analyses were performed by student's t-test. The values of $P<0.05$ were considered statistically significant.

Results and discussion

The essential oil of *Semenovia suffruticosa* was extracted by hydro distillation method. The oil yield(w/w%) was 0.77 based on dried weight of sample. The chemical composition of the oil was investigated using the GC-MS technique. Sixty compounds amounting

to about 84.99 % of the oil were identified, are: the major components found in the oil:cis-ocimene (19.31%) ,linalool (9.00 %),cinnamy valerate (8.19%), α-terpinolene (5.49%),6-amyl-α-pyrone (4.55%),isobutyl-isovalerate(3.71%),γ-terpinene(3.68%),α-bisabolol (2.93%) and unknown compounds (1.70%)(Table 1). The percentage of total monoterpenes and total sesquiterpene in the essential oil are 53.50and 7.92 respectively.

Table(1)

Chemical composition of the essential oil of *Semenovia suffruticosa*

Compound	RI	%
α-pinene	906	0.17
sabinene	946	0.39
1,8-Cineole	948	0.72
3-octanone	956	0.09
β-myrcene	960	0.46
isobutyl isobutyrate	977	3.71
δ-3-carene	983	0.17
α-terpinene	989	0.05
m-methylanisole	992	0.09
o-cymene	998	1.12
limonene	1002	0.24
cis-ocimene	1014	19.31
n-butyl isovalerate	1017	0.28
β-ocimene Y	1020	0.98
γ-terpinene	1035	3.68
p-cresol	1055	1.76
α-terpinolene	1065	5.49
linalool	1078	9.00
amyl isovalerate	1081	2.77
allocimene	1098	0.21
amyl valerate	1116	0.45
pulegone	1117	0.23
ethyldimethylthiophene	1146	4.64
1,8-metnthadien-4-ol	1153	0.17
4-terpineol	1155	0.09
p-cymen-8-ol	1160	0.27
α-terpineol	1166	0.11
trans-2,6-dimethyl-3,5,7-octatriene-2-ol	1169	0.14
p-allylanisole	1171	0.17
cis-2,6-dimethyl-3,5,7-octatriene-2-ol	1177	0.15

Compound	RI	%
cis-3-hexyl valerate	1201	0.63
hexyl isovalerate	1207	1.77
trans-3-hexyl valerate	1208	0.11
cis-4-decen-1-ol	1222	0.06
trans-4-decen-1-ol	1232	0.05
4-hydroxy-3-methylacetophenone	1250	0.34
eugenol	1318	0.09
α-copaene	1340	0.41
pentanoic acid,phenylmethyl ester	1350	0.85
methyleugenol	1358	2.19
Trans-caryophyllene	1374	0.31
unknown	1383	0.85
unknown	1412	0.22
trans-beta-farnesene	1419	0.28
unknown	1423	0.28
6-amyl -α -pyrone	1438	4.55
Granyl-isovalerate	1441	1.28
germacrene D	1458	0.25
zingberene	1462	0.12
lavanduilyl acetate	1470	0.20
bicyclogermacrene	1473	0.86
δ-cadinene	1494	0.79
cis- α-bisabolene	1506	0.47
germacrene B	1535	0.18
geranyl butyrate	1541	0.14
spathulenol	1555	0.14
geranyl isovalerate	1566	0.14
m-methylstyrene	1573	0.27
cinnamyl isovalerate	1589	0.27
valencene	1599	0.17
unknown	1621	0.35
cinnamyl valerate	1653	8.19
α-bisabolol	1658	2.93
angecin	1750	0.07
ficusin	1803	0.03
1-hxadecene	1827	0.05

Table (2)

Chemical composition of the essential oil of *Semenovia suffruticosa* by chemical class

Chemical class	Number of compounds	Percent of chemical class
Total monoterpenes	27	53.50
Hydrocarbon monoterpenes	12	32.37
Oxygenated monoterpenes	15	21.13
Total sesquiterpenes	13	7.92
Hydrocarbon sesquiterpenes	10	6.36
Oxygenated sesquiterpenes	3	1.56
Other hydrocarbon compounds	2	0.32
Other oxygenated compounds	17	18.61
Other compounds	1	4.62
Unknown compounds	4	1.70
Hydrocarbon terpenoids	22	38.73
Oxygenated terpenoids	18	22.69
Total terpenoids	40	61.42
Total without unknown compounds	60	84.99

Table (3)

The presented of Minimal inhibition Concentration of the essential oil from aerial parts of *Semenovia suffruticosa* for microorganisms

Microorganis	**Bacterial**				**yeast**	**Fungus**
Essential oil	*Salmonella typhi*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Candida albicans*	*Aspergillus niger*
Aerial parts	32 $\mu g.mL^{-1}$	32 $\mu g.mL^{-1}$	32 $\mu g.mL^{-1}$	8 $\mu g.mL^{-1}$	8 $\mu g.mL^{-1}$	32 $\mu g.mL^{-1}$

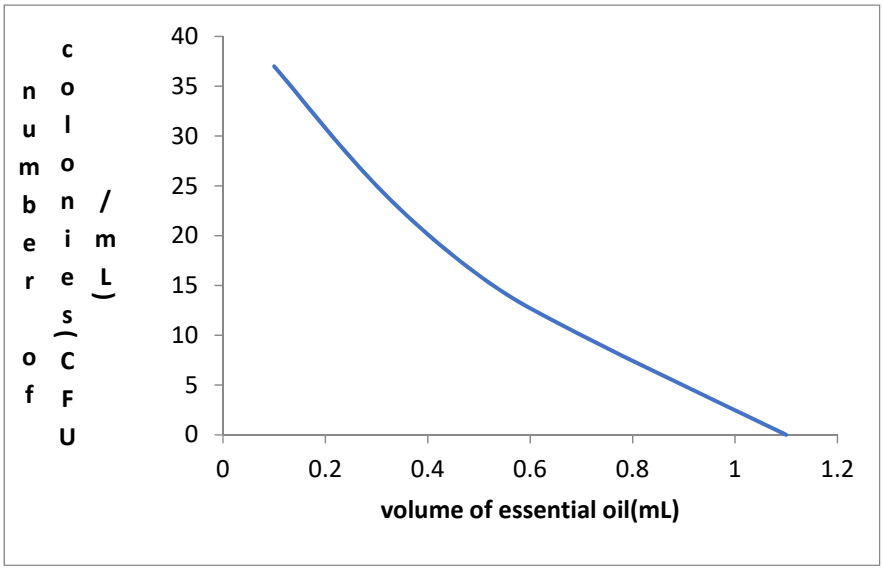

Figure (1): The variation of number of colonies as rate a function the variation of volume of the essential oil of *Semenovia suffruticosa*

The results of this examination are completely adapted with results of GC-MS technique of essential oil that confirms the existence of 22.69 % oxygenated terpeniods.In the essential oil of plant, this is oxygen-containing terpenoid compounds, in remarkable rate.So *Semenovia suffruticosa* essential oil has a good percent of total terpenoids (Table 2). Antimicrobial effects of this essential oil were exactly estimated and examined in laboratory.It,s sensitiveness(Minimal Inhition Concentration) to mentioned-micro-organisms in the following: *staphylococcus aureus*(8 $\mu g.mL^{-1}$), *Pseudomonas aeruginosa* (32 $\mu g.mL^{-1}$), *Salmonella typhi*(32 $\mu g.mL^{-1}$), *Escherichia coli* (32 $\mu g.mL^{-1}$), *Candida albicans*(8 $\mu g.mL^{-1}$), *Aspergillus niger*(32 $\mu g.mL^{-1}$), shows that the highest was recognized by comparing with standard the samples in the way of dilution, and minimum controlling the concentration was calculated *Staphylococcus aureus* and *Candida albicans* (Table 3).

The following microorganisms:*Staphylococcus aureus*(gram-positive bacteria), *Pseudomonas aeruginosa* ,*Salmonella typhi* and *Escherichia coli* (gram-negative bacteria) , *Candida albicans* (anti-yeast) , *Aspergillus niger* (antifungal), shows that the highest dilution of capable of the control on micro-organism has been *pseudomonas aeruginosa*. Comparison of the results showed that the antimicrobial feature of the oil is much greater oil due to its more combinations of oxygenated terpenoids, for instance linalool, 4- terpinol and alpha-terpineol**.**

Antimicrobial effects of this essential oil on water pollution were tested according to figure 1.In water polluted, H.P.C levels (number of colonies) are generally high. In water polluted under the direct the influence of different volumes of addition essential oil H.P.C bacteria concentration can be variable but are minimized through effective disinfection[15, 24].

Conclusion

In this study was to determine the essential oil antimicrobial effect was used of Agar dilution method.For the purposes of this standard six-microbial strains (a Gram-positive, four bacteria Gram in the negative,and two fungus strain) were used, use standard strains with the same identification code genetic microorganisms were identified to ensure that guarantee a reproducibility of results and possibility to compare the results of this

research is with the results of other researchers. So Gram-positive bacteria than Gram-negative bacteria and fungi, *Candida*. *albicans* is more sensitive to oil than the oil is more sensitive fungus *Aspergillus niger*. Lipid outer membrane of bacteria a Gram positive has pores that are called purines. This high-water conduit by multiple membrane proteins created and just let the free dissemination of hydrophilic molecules into the oil. So cannot easily hydrophobic properties pass of Gram-negative bacterial cell membrane. Anti-microbial experiments suggest that the essential oil of this plant can be used in filtration of water and waste water. Heterotrophic plat count bacterial growth in drinking-water shows this property[25].

List of Abbreviations

GC-MS: Gas chromatography/Mass spectroscopy technique **^{0}C:** Degrees a Celsius **mL:** Mili liter **min:** Minute **w/w%:** Weight / Weight percent **CFU.mL^{-1}**: Colony Forming Unit for liquids **Chemical class:** classification of compounds **MIC:** Minimal inhibition Concentration.

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التركيبات الكيميائية لزيت *semenoyasofroticosa* العطري وآثارها المضادة للميكروبات في الماء

علي رضا سردشتي، علي كوردي تسماندانى *

***جامعة سيستان وبالوشستان، كلية العلوم، قسم الكيمياء، زاهدان، إيران**

ملخص

وأفادت هذه الدراسة عن مركبات كيميائية ونشاط مضاد للبكتيريا للنفط الأساسي في براعم مصنع سيمينوياسوفروتيكا، الأصلي في إيران، وقد استُخرجت الزيوت الأساسية من البراعم بواسطة طريقة تقطير المياه وتم التحقيق فيها بواسطة الكروماتوغرافيا الغازية وتقنيات الكروماتوغرافيا الغازية المقترنة بالتقنيات الطيفية الكتلية. أظهرت النتائج أن المركبات الرئيسية التي بها أعلى نسب هي cis-ocimene 19.31 ٪، لينا لول 9٪، سينناميل فاليرات 8.19٪، إلفا تيربينولين 5.49٪، 6-إميل ألفا بيرون 4.55٪، وكانت النسبة لمركبات غير معروفة 0.85٪. كما تبين أن أعلى تركيز من النفط الأساسي على المبيضات ألبيكان وأدنى تركيز من النفط العطري قادرة على السيطرة على الكائنات الحية الدقيقة التالية: Staphylococcus aureus (8 µg/ml), Pseudomonas aeruginosa (32 µg/ml), Salmonella typhi (32 µg/ml), Escherichia coli (32 µg/ml), Candida albicans (8 µg/ml), Aspergillus niger(32 µg/ml) وزيادة الزيوت الأساسية بين العينات أدى إلى انخفاض في عدد مستعمرات الكائنات الحية الدقيقة.

الكلمات الدالة: Semenoyasofroticosa - مركبات النفط الأساسية – طريقة التخفيف أغار – عدد تقنية هيتروفويك .

تاريخ استلام البحث 2019/6/6 وتاريخ قبوله للنشر 2020/10/1.

Effectivity of Remdesivir and some compounds as therapeutic potential drugs for anti-SARS-CoV-2: in silico study

Jufrizal Syahri [1*], Beta Achromi Nurohmah[2], Emmy Yuanita[3]

[1]Department of Chemistry, Universitas Muhammadiyah Riau, Jalan Tuanku Tambusai Ujung, Pekanbaru, Indonesia.
[2]Department of Chemistry, Universitas Gadjah Mada, Jalan Kaliurang Sekip Utara Bulaksumur 21, 55281, Yogyakarta, Indonesia.
[3]Department of Chemistry, Universitas Mataram, Jalan Majapahit 62A, Mataram, Indonesia.
Corresponding authors' details; Jufrizal Syahri
Department of Chemistry, Universitas Muhammadiyah Riau, Pekanbaru, Indonesia, Tel/Fax. +62-761-35008. Email: jsyachri@umri.ac.id

ABSTRACT

Coronavirus Disease 2019 (COVID-19) pandemic by the infection of the new SARS-CoV-2 has been spread quickly worldwide. Since then, an effort has been made to find a therapeutic drug candidate to prevent and cure the infection. An **in silico** study is one of the most effective ways to do a screening of potential drugs by studying the interaction of the drug compounds with the protein target of the viruses theoretically. In this work, we reported the **in silico** study of 17 drugs candidates as anti-SARS-CoV-2 based on molecular docking. Three of the protein target used were crystal proteins of coronavirus with PDB ID of 2GX4, 6FV1, and 4LMT. The result showed that redemsivir showed the most promising drug candidate followed by hesperidin and chloroquine based on the CDOCKER energy and interaction formed form the molecular docking.

Keywords: Coronavirus, docking, *in silico*, Redemsivir, SARS-CoV-2.

1. INTRODUCTION

In late December 2019, an outbreak of pneumonia with an unknown cause was reported in Wuhan, China (Zhu et al., 2020; Hui et al., 2020; Lu et al., 2020). The World Health Organization (WHO) was then affirmed the outbreak as the Public Health Emergency of International Concern by the end of January 2020. The status was later declared as Pandemic on March 11, 2020, with the name of Coronavirus Disease 2019 (COVID-19), which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Guan et al., 2020; Huang et al., 2020; Wu et al., 2020; Paraskevis et al., 2020). The COVID-19 has spread quickly around the world, as of May 8, 2020, about 3,759,967 positive cases and 259,474 mortality have been reported globally (WHO, 2020).

Currently, there are no registered antiviral drugs for treating COVID-19 (Verma et al., 2020). Research and development of new preventive and therapeutic agents are needed immediately to prevent the uncontrolled number of deaths. Early prevention can be done by isolation or quarantine and followed by treatment according to the symptoms as to minimize the transmission (Zhang et al., 2020). Another possible solution to overcome this pandemic is to search and develop of broad-spectrum antiviral drugs by targeting the main protease of viruses (Xu et al., 2020). Repurposing some available drugs is one of the choices to search for the therapeutic agents for the treatment of COVID-19 as they reduce the time for drug development (Pandey et al., 2020).

Drug repurposing is an approach to search therapeutic agents for COVID-19 by using the current drugs that have been used in a different disease. Antimalarial agents (quinine, chloroquine, hydrochloroquine), antiviral

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(Lopinavir, ritonavir, remdesivir, ribavirin, Oseltamivir), and antibiotics (azithromycin, Tetracycline) are reported to be potential candidates for repurposing against SARS-CoV-2 (Pandey et al., 2020). Some flavonoids such as hesperidin, myricetin, curcumin, thymol, eugenol, quercetin with broad biological activity also have been studied as the therapeutics compound against COVID-19 (Adem et al., 2020; Ngwa et al., 2020; Zahedipour et al., 2020; Kulkarni et al., 2020; Colunga Biancatelli et al., 2020). Based on the literature, several flavonoid-based phytomedicines show a high binding affinity to the spike protein, helicase, and protease on the Angiotensin-converting enzyme (ACE) 2 receptor (Ngwa et al., 2020; Kulkarni et al., 2020).

Generally, coronavirus consists of structural (spike, membrane, envelope, and nucleocapsid protein) and non-structural proteins (Papain like protease/PLpro, Main protease/Mpro, and RNA-dependent RNA polymerase/RdRp) (Lavecchia and Fernandez, 2020). The chymotrypsin-like cysteine protease (3CLpro) is the main protease that can be found in all generations of coronaviruses such as SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV). The main protease 3CLpro has an essential role in replication and also gene expression of the virus (Lokhande et al., 2020). Thus, 3CLpro is proposed as the potential targets against SARS-CoV-2 (Chen et al., 2020). The crystal structures of coronavirus main protease such as 6LU7.pdb (Peele et al., 2020), 6M03.pdb (Singh and Florez, 2020), 1LVO.pdb (Hall and Ji, 2020), 6W01.pdb (Chikhale et al., 2020), and 5R7Y.pdb (Kumar et al., 2020), 6YB7 (Vijayakumar et al., 2020) have been reported as the protein target in the *in silico* study of some repurposed drug against COVID-19. Inhibition of some spike proteins with crystal structure 2GHV.pdb (Hall and Ji, 2020), 6M0J. pdb (Chikhale et al., 2020), and 6LZG.pdb (Vijayakumar et al., 2020) also have been studied using molecular docking. Spike protein is chosen as the target in the reduction of the infection of COVID-19 because it plays a role in the transmission and virulence of the virus (Hall and Ji, 2020). Meanwhile, nucleocapsid proteins in coronavirus can also be used as the target of the therapeutic drug because it is important in replication, transcription, and to build the structure (Tok and Tatar, 2017). Some studies have presented the *in silico* result of the crystal structure of SARS-CoV-2 nucleocapsid protein such as 6M3M.pdb (Kang et al., 2020; Bhowmik et al., 2020).

In this work, we performed *in silico* study of some commercial drugs such as remdesivir, chloroquine, hesperidin, and also proposed some candidate compounds against SARS-CoV-2 by targeting the main protease and nucleocapsid proteins of coronavirus. It has been reported that SARS-CoV-2 shows a 96% similarity with the protein sequences of SARS-CoV (Liu et al., 2020). Therefore, it is predicted that compounds with good activity against SARS-CoV would have good potential against SARS-CoV-2. The main protease of SARS-CoV (2GX4.pdb) (Xu et al., 2020), human coronaviruses (HCoV) NL63 (6FV1.pdb) (Zhang et al., 2020), and nucleocapsid protein (NP) of HcoV OC43(4LMT.pdb) (Chang et al., 2016) was used as the protein targets. The binding energy and interaction of the compounds against the coronavirus protein were evaluated to determine the best candidate compounds for SARS-CoV-2.

MATERIALS AND METHODS

Material

In this research, all of the docking studies were performed in a personal computer with Intel® Inside Core™ i7 processor (4 GHz, 8 GB RAM, 64-bit system type, and Windows® 10 Ultimate Operating system). The molecular structure of the compounds was drawn using ChemDraw Pro.15.0 software and the molecular docking was executed on the Discovery Studio 2016 (Accelrys, San Diego, USA) software.

Methods

Molecular docking was performed to the crystal proteins of coronavirus that were retrieved from Protein Data Bank (https://www.rcsb.org/) with PDB ID of

2GX4.pdb (resolution of 1.93 Å); 6FV1.pdb (resolution of 2.30 Å); and 4LMT.pdb (resolution of 1.71 Å). Protein and co-crystal ligands were separated first before the docking process in Discovery Studio software. Docking preparation of the protein was carried out by adding hydrogen atoms and adjusting the ionizable amino acids (residues) at default protonation (pH 7.4). The ligands were prepared to build 3-dimensional geometrics and to minimize the energy before the docking process. All of the docking processes were carried out following the standard protocol implemented from Discovery Studio software (Syahri et al., 2020).

During the docking process, the ligands were allowed to flex and the receptor was rigidly maintained. The docking tolerance from the docked conformer of ligand-receptor was set in 0.25 Å and the number of the nonpolar or polar hotspots in the receptor (to start the conformer fitting) was set at 500. The conformations of the ligands obtained from the docking process were fixed at 500 within the relative energy threshold of 20. Interactions of the ligand-receptor were observed in a grid box size of 90×90×90 with a grid map in Table 1. The validity of the docking methods was determined based on the *root mean square deviation* (RMSD) value <2.0 Å. The RMSD value obtained from the re-docking results of the co-crystal ligands to each protein of 2GX4, 6FV1, and 4LMT were 1.133; 1.850; and 1.890 Å, respectively.

Table 1. Grid map and radius applied in the docking process

PDB ID	X(Å)	Y(Å)	Z(Å)	Radius (Å)
2GX4	17.480279	1.480907	22.008395	9.00
6FV1	53.612154	12.144000	48.989128	10.00
4LMT	62.809955	15.413273	7.969364	9.00

RESULTS AND DISCUSSION

Preliminary stages using molecular docking is beneficial as it can predict the binding affinity between the compound (ligand) and protein. In this work, a molecular docking study of 17 compounds as a therapeutic candidate toward SARS-CoV-2 was performed to three different coronavirus crystal proteins such as 2GX4, 6FV1, and 4LMT (Table 1). The 2GX4 is a crystal structure of 3C-Like (3CL) protease inhibitor complex of SARS coronavirus and 6FV1 is the main protease from the human coronavirus NL63. Meanwhile, 4LMT is a crystal structure of nucleocapsid (N) protein of human coronavirus OC43.

Table 2 presented the CDOCKER energy (in kcal/mol) from the ligand-protein complex. Principally, the lower energy produced from the molecular docking process, the more stable interaction between the compounds (ligands) and protein targets. Remdesivir (**1**) showed the lowest CDOCKER energy (-64.4841 kcal/mol) among the 17 tested compounds against 2GX4 crystal strucure protein of SARS-CoV. This docking energy was the closest to the CDOCKER energy of the native co-crystal ligand (NOL) with the energy of -83.2383 kcal/mol. It also can be seen that remdesivir (**1**) showed the lowest CDOCKER energy to 6FV1 (-63.6467 kcal/mol) and 4LMT proteins (-53.0987 kcal/mol), compared with the other 16 compounds. It was revealed that both 2GX4 (from SARS-CoV) and 4LMT proteins (from HCoV-OC43) are categorized as β-coronavirus, while 6FV1 (from HCoV-NL63) is α-coronavirus. Thus, it can be interpreted that remdesivir has broad-spectrum antiviral activity. This result has a good agreement with some reported studies about the antiviral activity of remdesivir (Sheahan et al., 2020; Wang et al., 2020; Wu et al., 2020).

Hesperidin (**2**) displayed the best CDOCKER energy of -61.5854 kcal/mol to the 6FV1 protein, which closes to

the energy of remdesivir (**1**). Hesperidin was also placed as the second candidate of the drug for COVID-19 with lower docking energy against the crystal structure of 2GX4 (-59.5070 kcal/mol) and 4LMT (-50.1548 kcal/mol). The *in silico* study of hesperidin has been reviewed which is proposing the potential antiviral effect of hesperidin to the proein of SARS-CoV-2 (Bellavite and Donzelli, 2020). It has been published that hesperidin showed the suitable binding against the spike protein also lower binding energy to 3CLpro and Mpro of SARS-CoV-2.

Chloroquine (**3**) has the third-lowest CDOCKER energy of -46.2102 kcal/mol toward 6FV1 protein after remdesivir and hesperidin. Chloroquine (**3**) is widely reported to be the potential drug for the handling of COVID-19 (Colson et al., 2020). The interesting docking result of this compound (**3**) was that it displayed similar CDOCKER energy for all the tested proteins. This result encourages the previous study that proposed the use of Chloroquine (**3**) as an anti-SARS-CoV-2 drug candidate (Rebeaud and Zores, 2020; Devaux et al., 2020; Singh et al., 2020), although it still brings some debates (Touret and de Lamballerie, 2020; Jaffe, 2020). Chloroquine is still used as an antimalarial drug and it might lead to a serious threat concerning the expanding of the antimalarial drug resistance when it is not well-controlled.

Table 2. The CDOCKER Energy of some potential drugs

No	Compounds	CDOCKER Energy (kcal/mol)		
		2GX4	**6FV1**	**4LMT**
1	Remdesivir	–64.4841	–63.6467	–53.0987
2	Hesperidin	–59.5070	–61.5854	–50.1548
3	Chloroquine	–46.2048	–46.2102	–45.7560
4	Aminoalkylated Chalcone 1	–45.6236	–47.9247	–38.8552
5	Aminoalkylated Chalcone 2	–45.3461	–46.4737	–40.0853
6	Aminoalkylated Chalcone 3	–45.3258	–47.0167	–40.2087
7	Aminoalkylated Chalcone 4	–45.0230	–45.4285	–39.9402
8	Myricetin	–43.8472	–47.8862	–57.8559
9	Curcumin	–43.1526	–43.7372	–36.5257
10	Oseltamivir	–42.7158	–42.5152	–29.2826
11	Quercetin	–39.6190	–44.0359	–60.6573
12	Aminoalkylated Eugenol	–36.4853	–45.7011	–30.7630
13	Quinine	–34.0921	–38.1999	–32.9204
14	Ribavirin	–31.6247	–33.5869	–31.1901
15	Eleutherin	–28.4200	–36.1497	–28.3069
16	Eleutherinone	–26.1505	–32.1959	–26.0152
17	Xanthone	–19.5651	–25.2077	–19.4864

Aminoalkylated chalcone compound (**4-7**), which was reported to be active as antimalarial (Syahri, 2020), showed a CDOCKER energy that closes to the chloroquine (Table 2). This result indicates that the compound (**4-7**) also has the potential to be used as anti-SARS-CoV-2 drug candidates. Meanwhile, myricetin (**8**), curcumin (**9**), oseltamivir (**10**), and quercetin (**11**) exhibited moderate/medium CDOCKER energy to the tested

protein, except compounds (**11**) and (**10**) that displayed low CDOCKER energy of -60.66573 and -57.8559 kcal/mol to the 4LMT protein, respectively. It means that both of the compounds have a specific mechanism of action against the nucleocapsid protein. On the other hand, aminoalkylated eugenol (**12**), quinine (**13**), ribavirin (**14**), eleutherin (**15**), eleutherinone (**16**), and xanthone (**17**) were displaying higher energy for all the tested proteins.

Table 3 presented the interaction of the drug compounds (ligands) to the tested protein in the 2-dimension view. Based on the interactions formed, it can be seen that remdesivir (**1**) and hesperidin (**2**) have formed the highest number of hydrogen bonds to all the tested proteins. Remdesivir showed hydrogen bonds to the amino acid residues of Glu166, Gln189, His41, Cys145, and Asn142 (2GX4). The absence of H-bonds to His163 and Gly143 in the docking of **1**-2GX4 complex proposed the reason for the higher CDOCKER energy of remdesivir compared with the co-crystal ligand (NOL). The **1**-6FV1 complex presented the formation of H-bonds to Glu166, Gln164, Gly142, Pro189, Asn141, and Ile165 residues. Meanwhile, 2-D interaction of **1**-4LMT complex displayed the H-bonds to the Glu170, Arg122, Glu56, Tyr124, Ala171, Tyr 63, and Asp165 amino acid residues. Interaction of Hesperidin (**2**) with 2GX4 was formed via H-bonds to Glu166, Asn142, Leu141, Asp187, Met49, His164, and Met165 amino acid residues. Furthermore, H-bonds interaction of **2**-6FV1 can be seen to the Asn141, Glu166, Cys144, Phe139, His163, Ile140, Gln164, Thr47, and Ser190 residues. Lastly, **2**-4LMT complex showed H-bonds interaction to Ala171, Arg164, Asp169, Asp165, Pro166, Glu170, yr124, Tyr63, and Ser64 residues. This result was in agreement with the CDOCKER energy produced by these two compounds, indicating the higher potential as anti-SARS-CoV-2.

Chloroquine (**3**) has produced the most hydrogen bond to the 6FV1 protein compared with the other two tested proteins. It was predicted that chloroquine (**3**) has a specific inhibition mechanism of action to the main protease NL63 of human coronavirus. Meanwhile, compounds (**4-17**) were displayed fewer hydrogen bonds to all of the tested protein that indicates a lower potential as the candidate drugs for treating COVID-19.

Based on the *in silico* study, remdesivir, hesperidin, and chloroquine are highly recommended as candidates for anti-SARS-CoV-2. According to Wang et al. (2020), remdesivir and chloroquine are very effective in inhibiting COVID-2019, proved by the *in vitro* activity of both compounds with EC_{50} values of 3.7 and 10 μM, respectively. Ko et al. (2020) have also reported that remdesivir was a potential drug for COVID-19 therapy. Furthermore, remdesivir was previously reported to be active (*in vitro*) against the Ebola virus with an EC_{50} value of 0.06–0.14 μM (Warren et al., 2016), SARS-CoV with EC_{50} value 0.069 μM, and MERS-CoV with an EC_{50} value of 0.074 μM (Sheahan et al. 2017). Based on the clinical trials on monkey and mouse animals, remdesivir also has a very low toxicity level (Warren et al., 2016).

Hesperidin has been reported the be potential to halt the infection of SARS-CoV-2 based on computational screening (Utomo et al., 2020; Adem et al., 2020). In nature, the high content of hesperidin can be found in the peel of citrus fruits (Bellavite and Donzelli, 2020). As it is abundantly available in nature, hesperidin could be proposed to be used as a therapeutic compound for prevention and also the treatment of COVID-19. Moreover, hesperidin also showed a good safety profile and good tolerability according to human and animal studies (Bellavite and Donzelli, 2020). To conclude, this study strengthens the previous report that recommends remdesivir, hesperidin, and chloroquine as the drug candidates for the treatment of SARS-CoV-2.

CONCLUSION

This work proposed the potential of Redemsivir as anti-SARS-CoV-2 drug candidates based on the *in silico* studies by observing the CDOCKER energy and the interaction formed to the target protein from the molecular docking. Therefore it is expected that redemsivir could be

considered as the therapeutic drug against COVID-19.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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Table 3. 2-Dimension interaction of some potential drug compounds to the amino acid residues of protein 2GX4, 6FV1, and 4LMT

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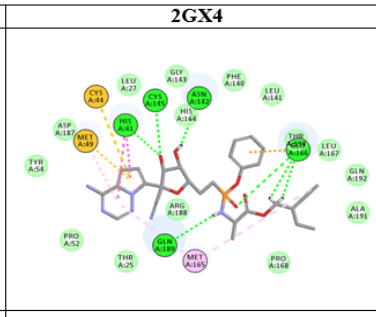
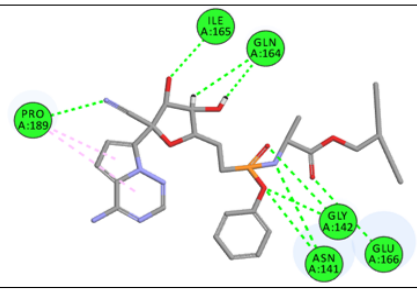
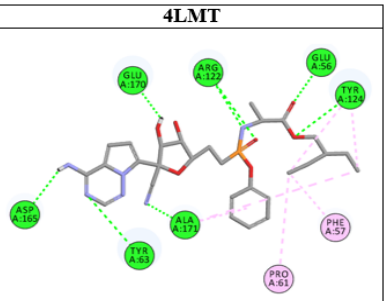
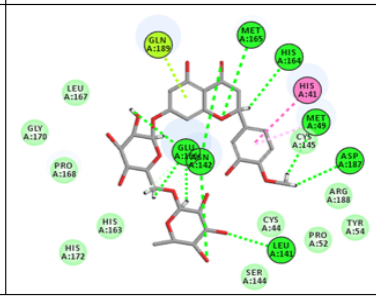
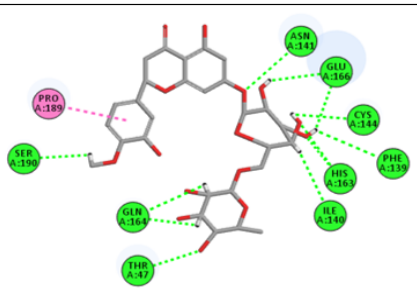
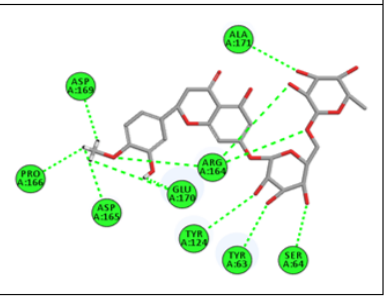
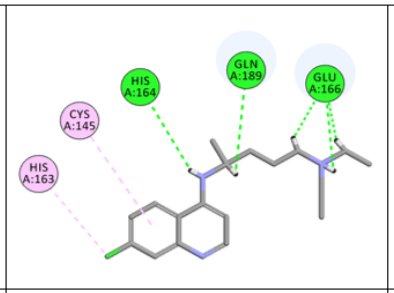
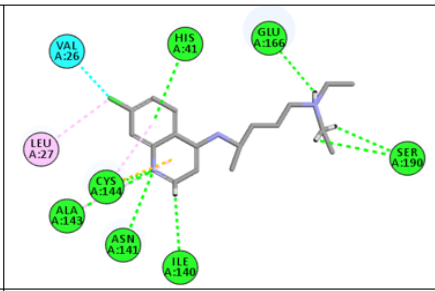
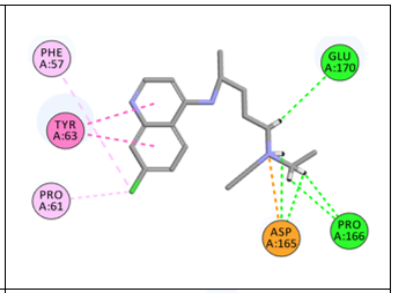
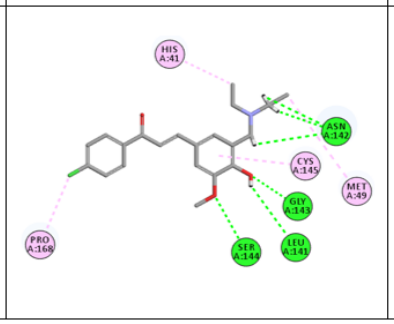
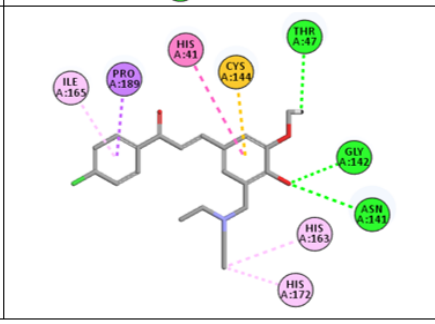
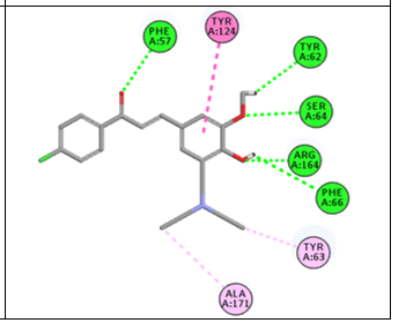
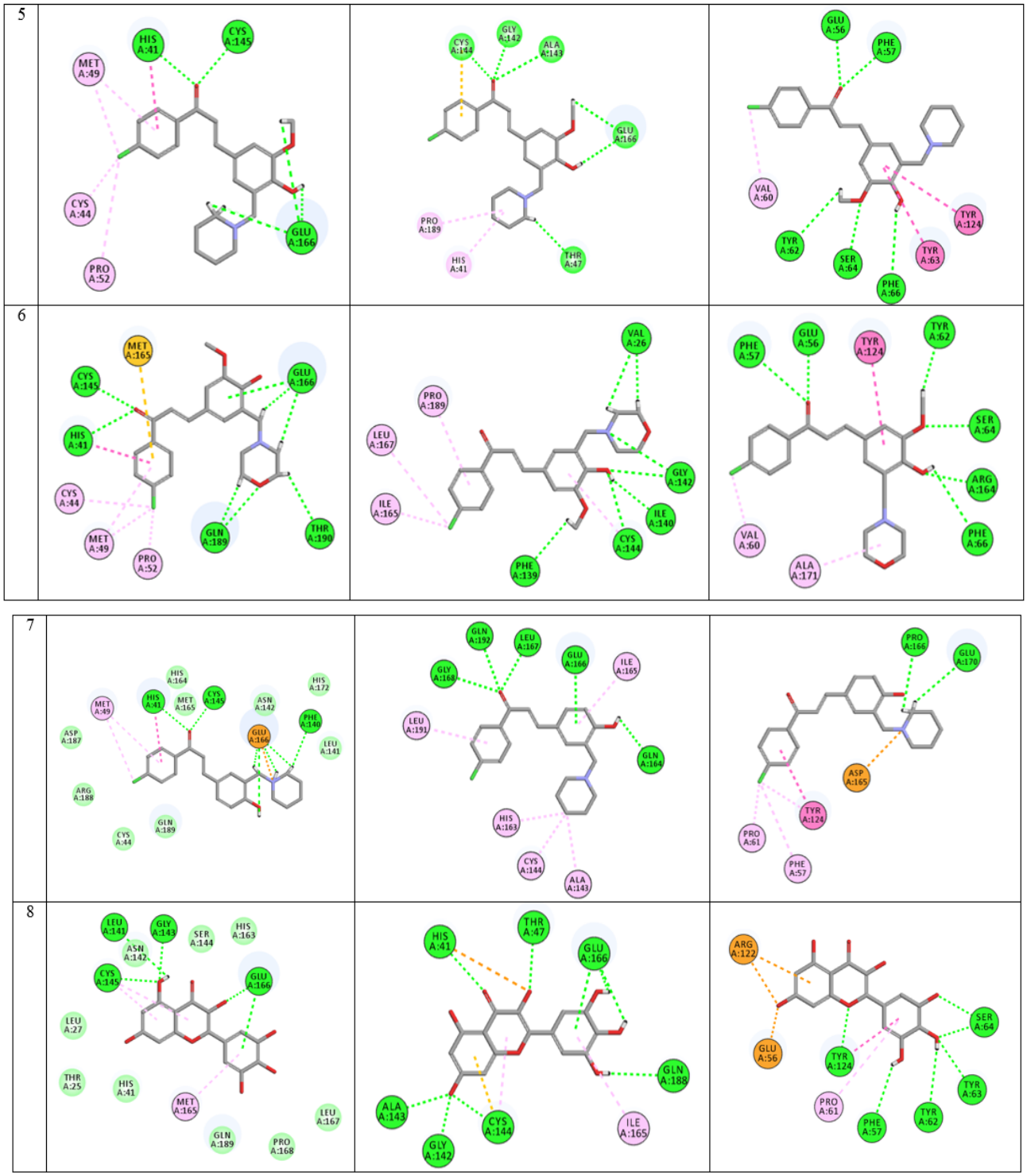
1			
2			
3			
4			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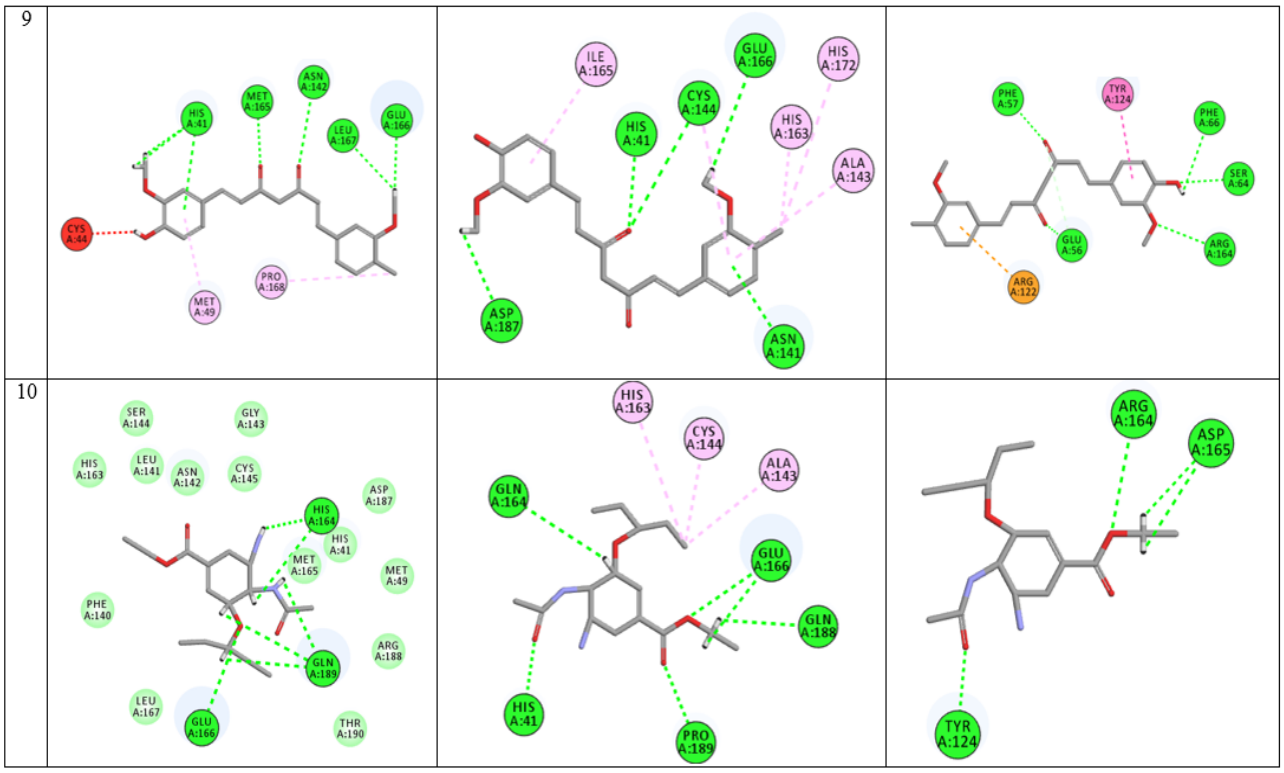
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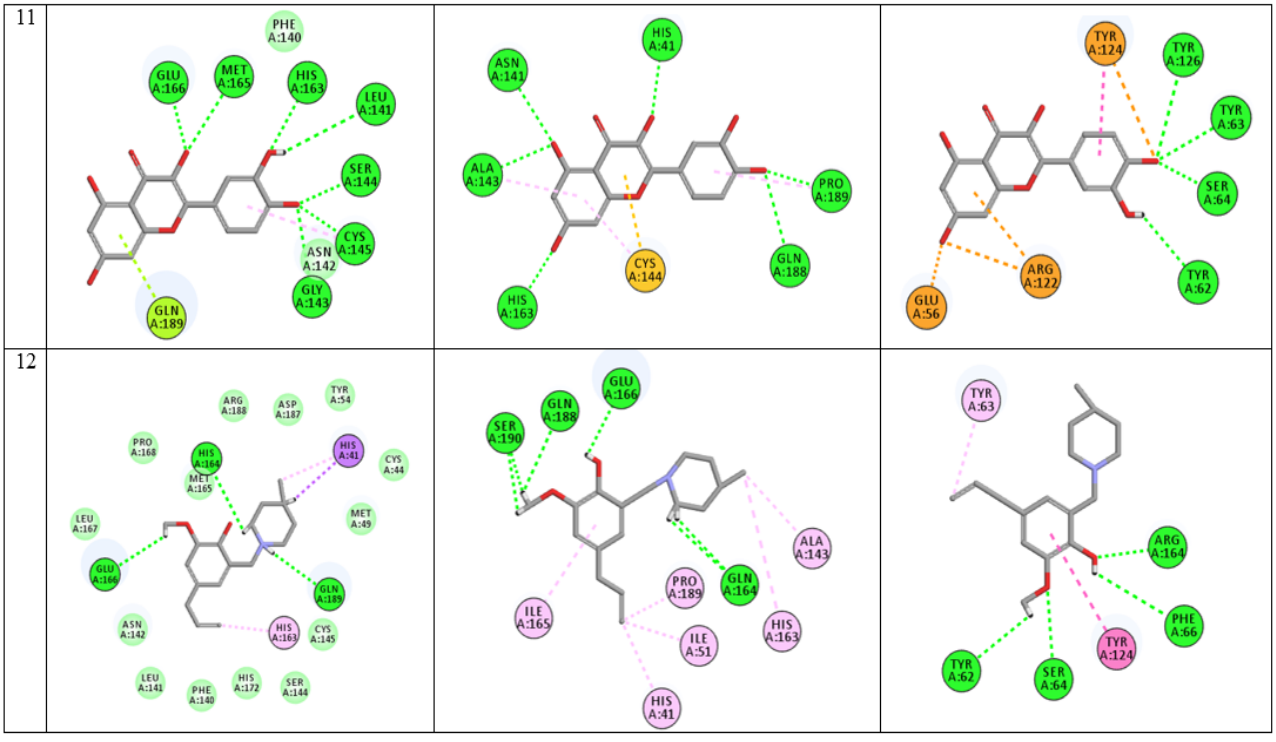
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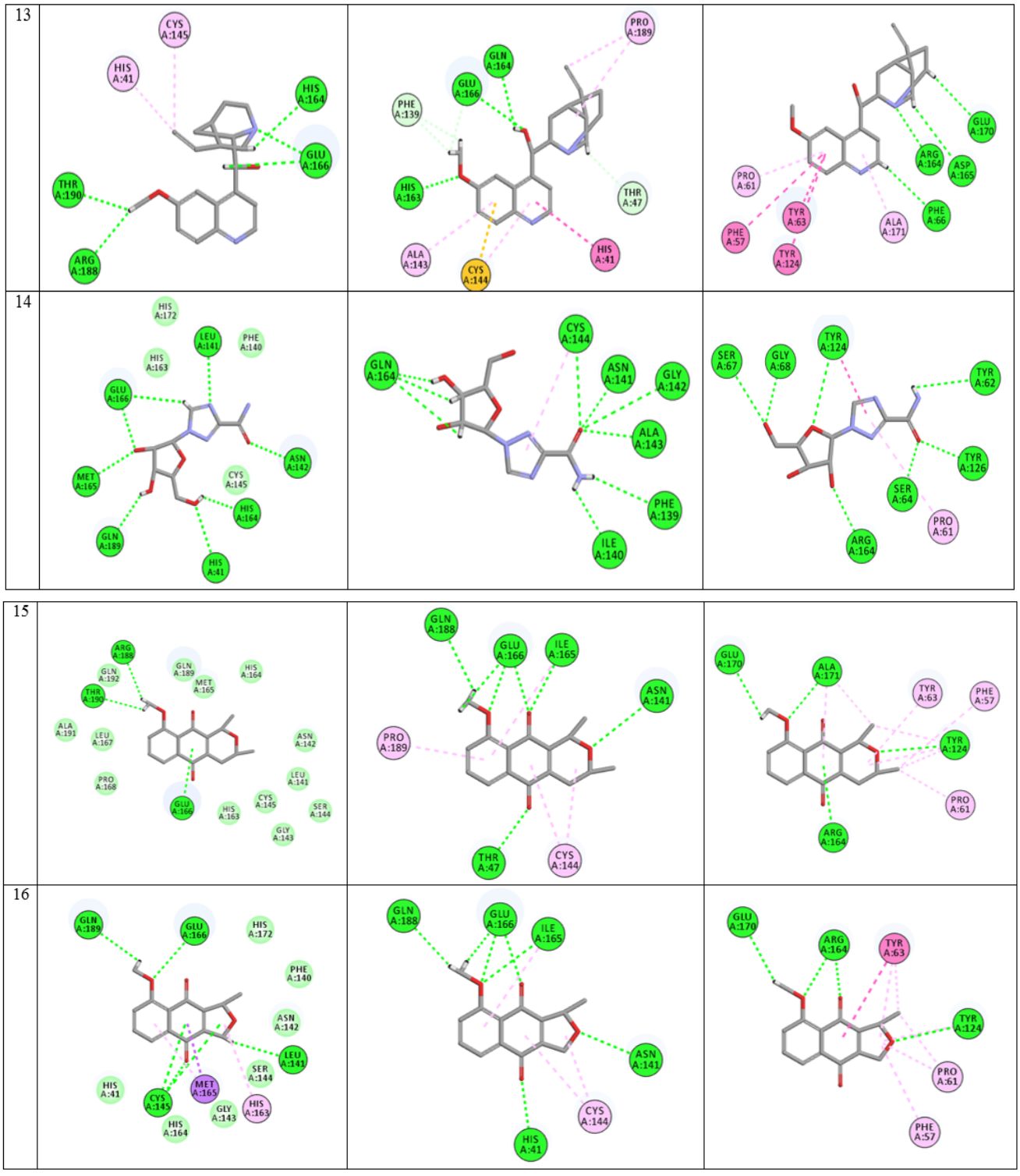
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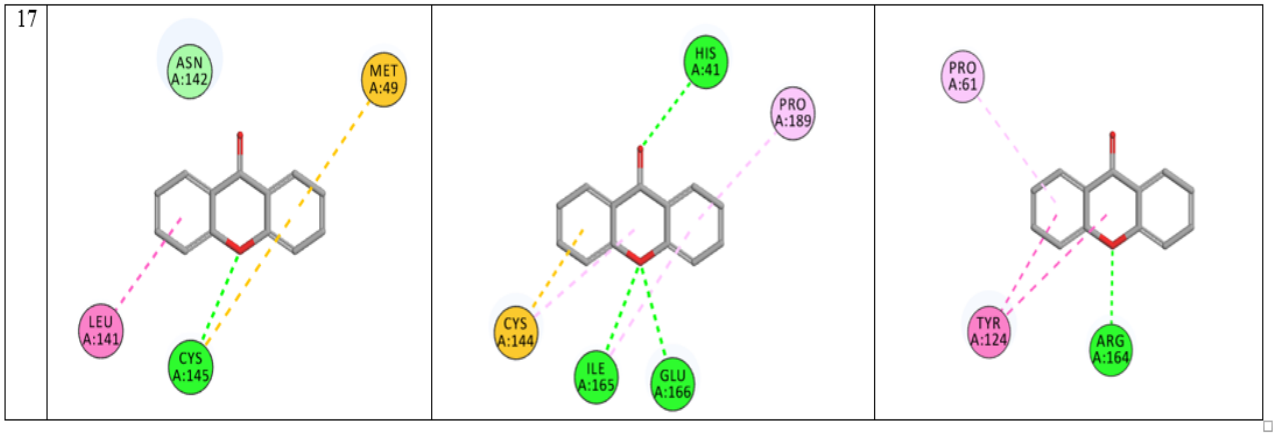

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فعالية ريمديسيفير والعديد من المركبات كأدوية علاجية محتملة لمقاومة SARS-CoV-2: دراسة في السيليكو

جوفريزال شهري[1]، بيتا أحرمي نور الرحمن[2]، إيمي يونيتا[3]*

[1]* قسم الكيمياء ، جامعة محمدية رياو ، جالان توانكو تامبوساي أوجونج ، بيكانبارو ، إندونيسيا.

[2] قسم الكيمياء ، جامعة غاجه مادا ، جالان كاليورانج سيكيب أوتارا بولاكسومور 21 ، 55281 ، جوغجاكرتا ، إندونيسيا.

[3] قسم الكيمياء ، جامعة ماتارام ، جالان ماجاباهيت 62 A ، ماتارام ، إندونيسيا.

ملخص

وباء فيروس كورونا 2019 (COVID-19) بالعدوى فيروس SARS-CoV-2 ينتشر بسرعة في جميع أنحاء العالم. منذ ذلك الحين، بذلت الجهود للبحث عن الأدوية المرشحة للوقاية من العدوى وعلاجها. تعد دراسات السيليكو واحدة من أكثر الطرق فعالية لإجراء فحص محتمل للأدوية من خلال دراسة التفاعلات النظرية لمركبات الدواء مع البروتينات المستهدفة الفيروسية. في هذا البحث ، أبلغنا عن دراسة في السيليكو لـ 17 عقارًا مرشحًا كمضاد لـ SARS-CoV-2 على أساس إرساء الجزيئي. كانت البروتينات المستهدفة الثلاثة المستخدمة عبارة عن بلورات بروتين فيروس كورونا برمز PDB 2GX4 و FV16 و LMT4. أظهرت النتائج أن ريمديسيفير كان أكثر الأدوية المرشحة الواعدة يليه الهسبريدين والكلوروكين بناءً على طاقة CDOCKER والتفاعلات المتكونة من نتائج إرساء الجزيئي.

الكلمات الدالة: كورونا فيروس، إرساء، السيليكو، ريمديسيفير، SARS-CoV-2.

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Determination of Epigallocatechin gallate (EGCG) and Caffeine in Domestic Tea Products using TLC-Densitometry

Febri Annuryanti, Indy Bustanyl Haq, Ali Nur Ad Deen, Djoko Agus Purwanto, Asri Darmawati*

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia.
**Corresponding author: asri-d@ff.unair.ac.id*

ABSTRACT

Background: Tea leaves are processed to produce black tea products and green tea products. Green tea is produced from the leaves without going through an enzymatic oxidation process while black tea undergoes a thorough enzymatic oxidation process. The enzymatic oxidation process can reduce the EGCG concentration in tea products.
Aim and objectives: This study aims to determine the concentration of EGCG and caffeine simultaneously in domestic green tea and black tea product of bag packaging using validated TLC-densitometry.
Methods: Silica gel GF254 TLC-plate was used as a stationary phase. The optimization of the mobile phase was carried out using a variety of organic solvents such as methanol, chloroform, ethyl acetate, n-butanol, and formic acid. The EGCG and caffeine were extracted from tea products with 40 mL of water at 80oC for 40 minutes with stirring and the analytes in water extract was re-extracted with ethyl acetate. The EGCG and caffeine in ethyl acetate extract were determined using validated TLC-densitometry.
Results: The mobile phase that optimally separate EGCG and caffeine with the nearest eluted analytes was chloroform: ethyl acetate: n-butanol: formic acid (20:10:7:3). The EGCG content in five green tea and black tea samples were in the range of (16.24-23.75) mg/g and (1.10-1.90) mg/g, respectively. Whereas the caffeine content in the same sample was in the range of (6.58-7.80) mg/g and (3.83-6.20) mg/g, respectively.
Conclusion: The TLC-densitometry method for the determination of EGCG and caffeine in the green tea and the black tea fulfilled the validation requirements for selectivity, linearities and accuracy. The precision for caffeine determination fulfilled the AOAC requirements, while the precision for EGCG determination of in green tea (5.23%) and black tea (7.87%) exceeded the AOAC requirement. TLC-densitometry of the extracts showed that the EGCG content in green tea was higher than in black tea, while caffeine content was relatively the same on both samples.
Keywords: EGCG, caffeine, green tea product, black tea product, TLC-Densitometry.

1. INTRODUCTION

Drinking tea is a part of the daily habits of some people. Nowadays, tea consumption tends to increase because of the various benefits of drinking tea, especially as nutraceutical. Information about the concentration of active substances that have a positive effect on tea products, such as catechin, is very important. Catechins are polyphenol group substances in tea leaves which have an antioxidant effect [(1)]. The main component of catechins is epigallocatechin gallate (EGCG) [(2)]. The EGCG was reported to have a various new founds effects such as adjunct in the treatment of hyper-cholesterol, obesity, cardiovascular and cancer [(3,4,5)]. The other catechin substances that have an antioxidant effect were Epigallocatechin (EGC), Epicatechin (EC) and Epicatechin gallate (ECG). Their molecular structure was shown in Figure 1. Besides EGCG, another substance with significant concentration in tea is caffeine [(2)]. Caffeine is a medium-strength CNS-stimulant, which give a fresh-tasting effect. However, caffeine causes palpitations and difficulty in sleeping for some sensitive people.

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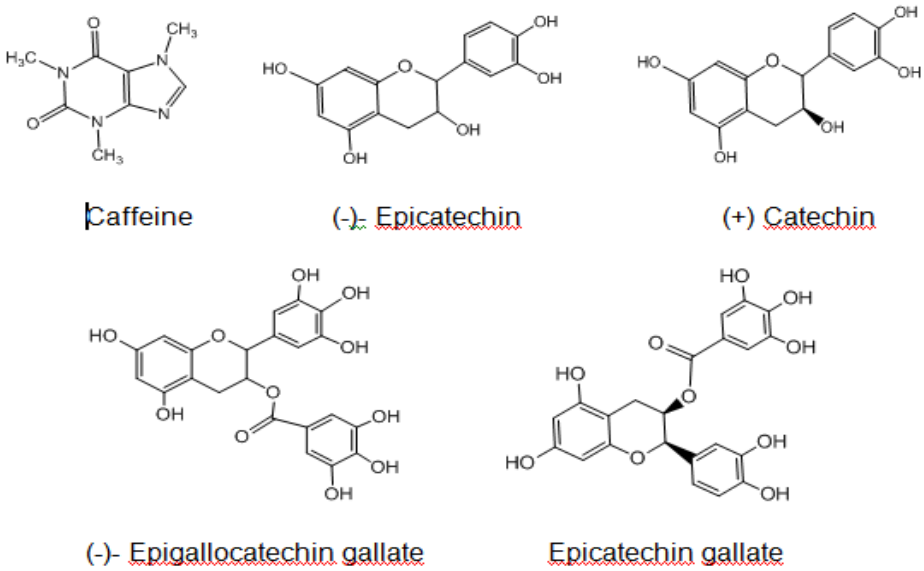


Figure (1): Molecular structures of caffeine, EGCG, and the other catechin derivate

The processing of tea leaves can produce green tea and black tea. Green tea is produced without going through an enzymatic oxidation process, while black tea undergoes a complete enzymatic oxidation process. The enzymatic oxidation process can reduce the EGCG concentration in the end products [3]. In China, the EGCG concentration in green tea products was reported higher (1.1-62.4 mg/g) than EGCG concentration in black tea (1.99-3.79 mg/g) [1,4]. But the caffeine concentration is relatively the same (21.6-29.6 mg/g). The simultaneous determination of EGCG and caffeine in Indonesian green tea products and black tea products was not well informed.

The EGCG concentration in tea leaves was influenced by species, age of the leaf, climate, and horticultural practices [5]. Different EGCG analysis methods can produce a variation of EGCG levels. The similar structure of catechin derivatives makes a certain method difficult to distinguish or separate each analytes signal. High-performance liquid chromatography (HPLC) [6,7,8] or TLC-densitometry [9] is the common method used for the determination of EGCG and caffeine simultaneously. The National Standardization Agency of Indonesia (SNI 3945:2016) stated the minimum requirement for polyphenol content in green tea products is 15% [10]. The SNI-3945:2016 does not mention the requirements for EGCG or caffeine concentrations in green tea [10] or black tea [11]. Nevertheless, some imported tea products have listed the content of EGCG and caffeine in their brochures. Therefore, to maintain quality (including product standardization), loyalty and safety of consumers, the content of EGCG and caffeine in tea products need to be mentioned in the packaging/brochures.

This study aims to validate the TLC-densitometry method for the determination of EGCG and caffeine concentration simultaneously in green tea and black tea products, in terms of selectivity, linearity, accuracy, and precision. The validated TLC-densitometry method then used to determine the EGCG and caffeine content in green tea and black tea products. Five samples of each type of tea product were selected based on consumer preference data.

2. MATERIALS AND METHODS

2.1 Instrument and apparatus

CAMAG TLC Scanner 4, CAMAG chromatography chamber 20.0 x 10.0 cm, Win CATS software, UV CAMAG lamp. Mettler Toledo microbalance (d = 0.001

mg), Ohaus analytical balance (d = 0.001g), Socorec micropipette (1.0 mL), Eppendorf micropipette (0.1-1.0 mL), capillary micropipette 2.0 µL of Blaubrand IntraEnd, hot plate Ika C-mag, magnetic stirrer, and Hectic Zentrifugen EBA-20 centrifuge.

2.2 Material

Unless otherwise specified, the following substances were of pro analysis (pa) quality of E. Merck. Standard EGCG pharmaceutical-grade purity (Xi'an Rhongseng Co, LTD), caffeine, ethyl acetate, chloroform, n-butanol, formic acid, isopropanol, and silica-gel GF254 TLC plate.

Five samples of green tea bag products were labelled as Ga®, Je®, Sa®, SS®, To®; and five samples of black tea bag products were labelled as Po®, Sa®, SS®, Du®, To®.

2.3 Sampling

The five most popular brands of green tea and black tea of Indonesia domestic products that sold in East Surabaya were purchased randomly from supermarkets. All samples registered with "MD" code (legally marketed in Indonesia).

2.4 Procedure

2.4.1 Preparation of EGCG and caffeine standard solution

Stock solutions were prepared by dissolving accurately weighed 10 mg of EGCG and caffeine in 10 mL of ethyl acetate. Calibration standards were prepared by diluting the stock solution with the same solvent to obtain various EGCG and caffeine concentrations. Two microliters of standard solutions were spotted on silica-gel GF254 TLC plate.

2.4.2 Preparation of EGCG and caffeine extract

Tea powder was removed from green or black tea bag samples and then mixed homogeneously. Two grams of the tea powder was accurately weighed and transferred into beaker glass. The sample was added with 40 ml of water at 80ºC, then stirred using a magnetic stirrer over a water bath at 80ºC for 40 minutes. Subsequently, the sample was allowed reach room temperature before transferred quantitatively into a 50 mL volumetric flask, added with water until the mark and shaken homogeneously [12]. The aliquot mixture was transferred to a centrifuge tube and centrifuged at 2500 rpm for 10 minutes. An aliquot of 25.0 mL of supernatant was extracted with 10 mL of ethyl acetate twice. The ethyl acetate phase was collected in a 25 mL volumetric flask and added with the same solvent until the mark*. Finally, 2.0 µL of the solution was spotted on a silica-gel GF254 TLC plate.

*The black tea sample needed to be concentrated by evaporating 10.0 ml of the ethyl acetate extract with nitrogen (N2) gas flow. The residue then re-dissolved with 2.0 mL of ethyl acetate before spotted on silica GF254 TLC plate.

2.4.3 Selectivity and specificity test

Selectivity parameters were R_f (retardation factor), R_s (Resolution) and peak purity. The specificity parameter was peak identity. Various compositions of mobile phases were tested to obtain a good resolution (Rs) among EGCG, caffeine and other analytes in the samples. A good R_s ideally is more than 1.5[13]. However, the R_s value of 1.2 is also acceptable because the separation between the two peaks is relatively completed [14]. Acceptance criteria for peak purity and peak identity is when r value is more than 0.9900[15].

2.4.4 Linearity test

Two microliters of EGCG and caffeine standard solutions in the range concentration of 100 ppm, 200 ppm, 300 ppm, 400 ppm, dan 500 ppm were spotted on TLC plate and eluted with a mixture of chloroform: ethyl acetate: formic acid: n-butanol (20:10:3:7) as mobile phase. The developed spots scanned with a densitometer and detected by UV light detector at a wavelength of 275 nm. The equation of linear regression was Y = bx + a. A good coefficient correlation (r) between analytes concentration and the area is accepted when the r value is > 0.99 and the coefficient of function variation (Vxo) < 5%.

2.4.5 Accuracy and precision

Accuracy was determined based on the recovery value of the standard addition. An aliquot of 5.0 mL of ethyl

acetate extract of green tea that had been obtained on step 2.4.2 was added with EGCG and caffeine standard of 80%, 100% and 120% of analytes concentration. Two microliters of recovery solution were spotted on the TLC plate and eluted using the selected mobile phase. The standards of EGCG and caffeine were added into the black tea extract that had been concentrated. Percentage recovery was calculated as follows.

$$\text{Recovery (\%)} = \frac{\text{(Total analyt concentration obtained)}}{\text{sample concentration + standard added concentration}} \times 100$$

Precision value was determined using the equation below:

Precision (%) = (Standard deviation of analytes recovery /mean) x 100

2.4.6 Determination of EGCG and caffeine in the tea bag sample

Two microliters of ethyl acetate extract that had been obtained on step 2.4.2 was spotted on the TLC plate. After eluted using a mixture of chloroform: ethyl acetate: formic acid: n-butanol (20:10:3:7) as mobile phase, the spots were scanned using a densitometer and detected at λ 275 nm. The analytes concentration in the sample spot was determined based on the calibration curve of EGCG and caffeine.

3. RESULTS AND DISCUSSION

3.1 Selectivity

Different kinds of mobile phases were analyzed and the mixture of chloroform: ethyl acetate: formic acid: n-butanol (20: 10: 3: 7) was selected as mobile phase. The selected mobile phase optimally separated the EGCG and caffeine from other nearby substances with the R_s value in the range of 0.88-1.57. The range of the R_s value among substances caused by the variation of the matrix sample in five samples of green tea products and black tea products.

The black tea extract showed more complex chromatogram compared to the green tea extract. Additional peaks in the black tea chromatogram indicates that there is a possibility of the formation of new compounds that occurs during the long manufacturing process of black tea, which is involving enzymatic oxidation processes. The three-dimension chromatogram profiles of EGCG, caffeine and other substances in the green tea and black tea extracts were shown in Figure 2.

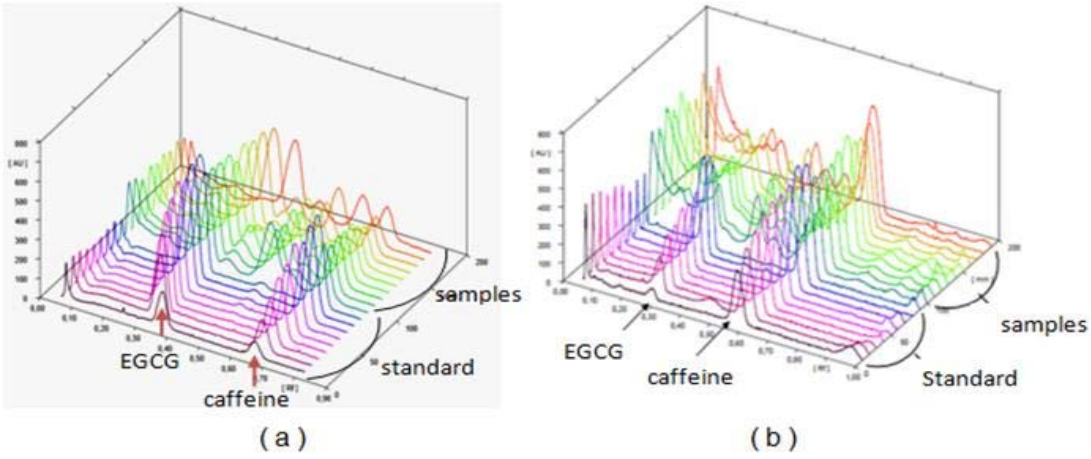


Figure (2): Three-dimension chromatogram of EGCG and caffeine in the green tea extract (a) and black tea extract (b)

The EGCG and caffeine peaks in black tea were not completely separated from the adjacent peaks (Rs < 1.5). Therefore, the area of EGCG and caffeine was measured based on a modified baseline, 'valley to valley' adjustment program. The modified baseline made the area of EGCG or caffeine not contaminated by the area of the nearby substances and resulted in smaller areas than it should be. As shown in Figure 3c, the peak of EGCG or caffeine was not completely separated from the closest substances. The two-dimension (2D) chromatogram profile of the tea extract showed in Figure 3. The measurement of the area based on 'base to base' adjustment, made the area of EGCG or caffeine higher than it should be because of an additional area of the nearby substances that were not completely separated. If the area obtained by 'base to base' adjustment converted to a concentration based on a completely separate EGCG or caffeine (Figure 3a) in the calibration curve, it would result in a concentration that was higher than it should be.

Peak identity, as a parameter to ensure the similarity between suspected EGCG or caffeine peak in the sample with the standard, showed a good correlation (r > 0.9900) (Figure 4). The peak identity was determined based on the match factor between the UV spectra of EGCG or caffeine peak in sample and standard at the same retention factor (R_f). It can be concluded from the peak identity results that the suspected EGCG or caffeine in samples were the same as EGCG and caffeine standards.

The maximum wavelength of EGCG and caffeine were 275 nm and 279 nm, respectively (Figure 4). The wavelength of measurement was set at 275 nm because of its sensitivity.

Peak purity determined by spectra coefficient correlation ($r_{spectra}$) between the beginning of the peak with the middle peak ($r_{s,m}$) and r between the middle peak with the end of the peak ($r_{m,e}$). The peak-purity test result of EGCG or caffeine in green tea or black tea samples showed an r value > 0.9500, which means that sample-peaks were not contaminated (data not shown) [15].

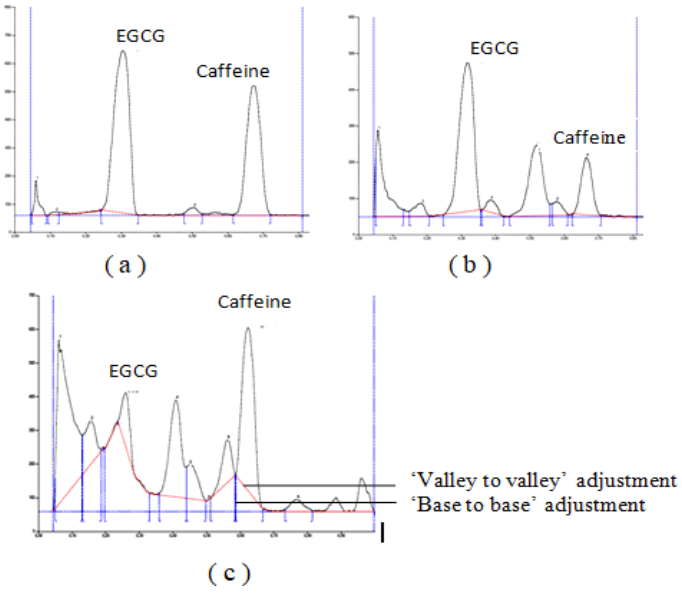
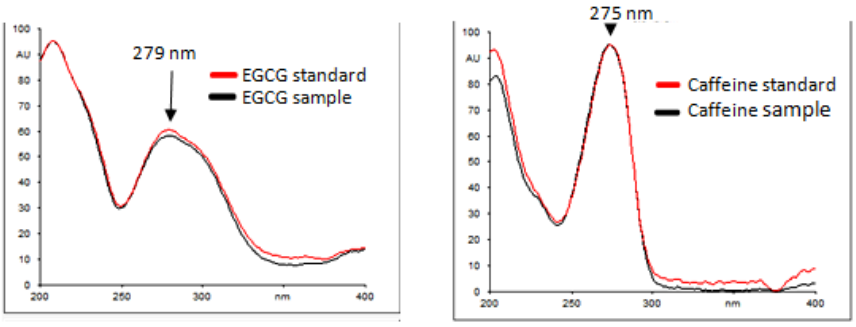


Figure (3): The two-dimension (2D) chromatogram of a mixture of EGCG and caffeine standard (a), green tea sample coded Sa®(b) and black tea sample coded Po® (c)



(a) (b)

Figure (4): The UV spectra profile of the peaks of EGCG (a) and caffeine (b) in the sample (coded Sa®) overlaid with the standard spectra of EGCG and caffeine

3.2 Linearity

The result of EGCG and caffeine linearity test showed that the detector response had a linear relationship with the analytes in the analytical range concentration. The linear equation as included in Table 1.

Table (1)
The result of linearity test of EGCG and caffeine

EGCG		Caffeine	
Range of concentration (ppm)	Parameter	Range of concentration (ppm)	Parameter
403.4 – 1814 (Green tea)	r = 0.9974 r^2= 0.9948 Vxo = 3.85 % Y =11.008 x + 6244.6	82.80 – 745.2 (Green tea)	r = 0.9994 r^2 = 0.9988 Vxo = 2.20 % Y = 23.455 x + 3638.6
81.20 – 568.40 (Black tea)	r =0.9991 r^2= 0.9982 Vxo = 3.02 % Y = 15.75 x - 365.31	80.40 – 643.20 (Black tea)	r = 0.9986 r^2 = 0.9972 Vxo = 3.77% Y = 25.50 x + 3027

3.3 Limit of detection (LOD) and limit of quantitation (LOQ)

Based on the standard deviation of the linear equation function of analyte in the range concentration of (39.32-147.4) ppm for EGCG and (19.67-147.5) ppm for caffeine, the LOD and LOQ of EGCG were 7.96 ppm and 26.52 ppm, respectively. While the LOD and LOQ of caffeine were 5.59 ppm and 18.62 ppm, respectively. As a note, all these concentrations were obtained based on the spot area of 2 µL solution.

3.4 Accuracy and precision

The percentage recovery of EGCG in green tea and black tea were (95.30 ± 5.03) % and (99.44 ± 7.83) %, respectively (Table 2). An independent *t-test* showed no significant difference of EGCG concentration in green tea and black tea samples (P = 0.301 > 0.05%).

The percentage recovery of caffeine in green tea and black tea was shown in Table 3. The independent *t-test* of caffeine recovery in green tea and black tea revealed that no significant difference in caffeine concentration in both samples (P = 0.423 > 0.05%).

The result of accuracy test for determination of EGCG and caffeine simultaneously in tea products using TLC-densitometry fulfilled the AOAC requirement [13] for analytes concentration of 0.1% and 1%, which is 90-108% and 92-105%, respectively.

The AOAC requirement for the precision of 1% analyte concentration in the sample is 4%, whereas for 0.1% of analyte concentration in the sample is 6%. The precision result of EGCG in green tea (5.23%) and black tea (7.87 %) was out of the required range. However, the precision of caffeine in green tea (2.91%) and black tea (5.50%) fulfilled the AOAC requirement.

Table (2)
Accuracy and precision of EGCG determination in tea sample

Samples and EGCG standard addition		The EGCG in the analyzed solution				Mean (%)	CV (%)
		Sample (mg)	Standard Addition (mg)	Sample + addition (mg)	Recovery (%)		
Green tea	80%	4.002	3.305	6.737	92.20	95.30 ± 5.03	5.23 %
		4.358	3.403	7.546	97.23		
		4.159	3.287	6.661	89.46		
	100%	4.545	4.249	8.433	95.89		
		3.824	4.276	8.243	101.7		
		4.543	4.295	8.955	101.3		
	120%	4.002	5.004	8.200	91.05		
		3.824	5.052	8.853	99.74		
		4.159	5.106	8.253	89.08		
Black tea	80%	0.1493	0.1559	0.2934	96.13	99.44 ± 7.83	7.87 %
		0.2847	0.1647	0.4756	105.8		
		0.0927	0.1677	0.2711	104.1		
	100%	0.1493	0.1943	0.3413	99.34		
		0.2847	0.2064	0.5398	109.9		
		0.0927	0.2017	0.2473	84.01		
	120%	0.1493	0.2341	0.3523	91.89		
		0.2847	0.2505	0.5432	101.5		
		0.0927	0.2417	0.3421	102.3		

Table (3)
Accuracy and precision of caffeine determination in tea sample

Sample and Caffeine standard addition		The caffeine in the analyzed solution				Mean (%)	CV (%)
		Sample (mg)	Standard Addition (mg)	Sample + addition (mg)	Recovery (%)		
Green tea	80%	1.171	1.141	2.251	97.36	98.18 ± 2.86	2.91 %
		0.942	1.219	2.274	105.23		
		1.102	1.224	2.258	97.07		
	100%	1.171	1.478	2.531	95.55		
		0.942	1.458	2.322	96.75		
		1.253	1.418	2.615	97.89		
	120%	1.513	1.592	3.088	99.45		
		0.942	1.621	2.471	96.41		
		1.189	1.641	2.772	97.94		
Black tea	80%	0.2524	0.1700	0.4237	100.23	96.59 ± 5.52	5.40 %
		0.2695	0.1604	0.4277	99.47		
		0.1169	0.1750	0.2714	92.99		
	100%	0.2524	0.1885	0.4101	93.00		
		0.2695	0.2150	0.4808	99.24		
		0.1169	0.2206	0.3326	98.55		
	120%	0.2524	0.2500	0.5132	101.96		
		0.2695	0.2566	0.4493	85.39		
		0.1169	0.2814	0.3911	98.20		

3.5 Determination of EGCG and caffeine in green tea and black tea products

The simultaneous determination of EGCG and caffeine in green tea and black tea products were shown in Table 4 and Table 5. Data were obtained from triplicate experiments.

The EGCG concentrations were calculated and reported as in the dry sample. The water content of green tea and black tea samples was in the range of 6.94-8.45 %, whereas the water content requirements for green tea and black tea according to SNI are ≤ 7% [10,11]. In this study, we used the gravimetric method (heating 105°C in the oven for 1 hour) for determination of the water content instead of Karl Fisher reagent as stated in SNI. Therefore, there was a possibility that other substances besides the water in the samples that also evaporated.

The EGCG concentration in green tea samples was (16.24-23.75) mg/g, while EGCG concentration in black tea was (1.10-1.90) mg/g. This value was in accordance with the range of EGCG concentration as mentioned in the reference [2]. Nevertheless, the caffeine concentration in green tea samples (6.58-7.80 mg/g) and black tea samples (3.83-6.20 mg/g) was lower than caffeine concentration as reported in the reference (21.6-29.6) mg/g [2]. Low caffeine concentration in domestic tea products is preferred and safer for caffeine-sensitive tea consumers.

Table (4)
The EGCG and caffeine concentration in Indonesian green tea product

Samples	EGCG (mg/g)		Caffeine (mg/g)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Ga®	23.37 ± 0.06	0.26	7.44 ± 0.27	3.65
Je®	23.75 ± 0.18	0.76	7.73 ± 0.26	3.38
Sa®	22.85 ± 0.41	1.79	7.50 ± 0.27	3.62
SS®	17.97 ± 0.67	3.73	6.58 ± 0.12	1.89
To®	16.24 ± 0.23	1.42	7.80 ± 0.48	6.10

Table (5)
The EGCG and caffeine in Indonesian black tea product

Samples	EGCG (mg/g)		Caffeine (mg/g)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Po®	1.90 ± 0.02	0.90	3.83 ± 0.32	8.76
Sa®	1.27 ± 0.04	2.96	3.91 ± 0.08	2.28
SS®	1.66 ± 0.05	2.77	5.95 ± 0.59	9.87
Du®	1.42 ± 0.06	4.29	5.38 ± 0.53	10.69
To®	1.11 ± 0.06	5.24	6.20 ± 0.15	2.46

4. CONCLUSION

The TLC-densitometry method for simultaneous determination of EGCG and caffeine in the green tea and black tea products met the validation requirements in terms of selectivity, linearity, and accuracy. The precision results for caffeine levels met the requirements, while the precision of this method for EGCG determination in green tea (5.28%) and black tea (7.87%) was outside the AOAC requirement.

The EGCG concentration in green tea products (16.24-23.75 mg/g) was relatively higher than in black tea (1.10-1.90 mg/g). While the caffeine concentration in green tea (6.58-7.80 mg/g) and black tea (3.83-6.20 mg/g) were relatively the same.

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Conflict of interest

No conflict of interest was declared by the authors.

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قياس الكثافة والكافيين في منتجات الشاي المنزلية باستخدام TLC-

اسري داريماواتي ، فبني النور ينتي ، عندي بستانيل حق ،علي نور الدين ، جوكو اكوس فوروانطا *

قسم الكيمياء الصيدلية ،كلية الصيدلة ،جامعة إيرلانجا ،سورابايا ،إندونيسيا.

*المؤلف المراسل asri-d@ff.unair.ac.id

ملخص

الخلفية: تتم معالجة أوراق الشاي لإنتاج منتجات الشاي الأسود ومنتجات الشاي الأخضر. الشاي الأخضر هو نتيجة معالجة أوراق الشاي بدون عملية أكسدة إنزيمية. بينما يصنع الشاي الأسود عن طريق الأكسدة الأنزيمية الكاملة لأوراق الشاي. يمكن لعملية الأكسدة الأنزيمية أن تقلل من تركيز **EGCG** في المنتج النهائي. الهدف والأهداف: كان الهدف من هذه الدراسة هو تحديد تركيز **EGCG** والكافيين في وقت واحد في الشاي الأخضر المحلي ومنتجات الشاي الأسود لتغليف الأكياس باستخدام مقياس كثافة **TLC** المعتمد. الطريقة: تم استخدام هلام السيليكا **GF254 TLC-plate** كمرحلة ثابتة ومزيج من تركيبة مختلفة من المذيبات العضوية مثل الميثانول ، الكلوروفورم ، أسيتات الإيثيل ، **n**– بيوتانول ، حمض الفورميك كمرحلة متحركة. تم استخراج **EGCG** والكافيين من منتجات الشاي مع 40 مل من الماء عند 80 درجة مئوية لمدة 40 دقيقة مع التحريك. تمت إعادة استخلاص المواد التحليلية في مستخلص الماء باستخدام أسيتات الإيثيل. تم تحديد **EGCG** والكافيين في مستخلص أسيتات الإيثيل باستخدام مقياس كثافة **TLC** المعتمد. النتائج: الطور المتحرك الذي يفصل على النحو الأمثل **EGCG** والكافيين مع أقرب مادة تحليلية مزيلة هو الكلوروفورم: أسيتات الإيثيل: **n**– بيوتانول: حمض الفورميك (20: 10: 7: 3) كان محتوى **EGCG** في خمس عينات من الشاي الأخضر والشاي الأسود في حدود (16.24–23.75) مجم / جم و (1.10–1.90) مجم / جم على التوالي. في حين تراوح محتوى الكافيين في نفس العينة بين (6.58–7.80) ملجم / جم و (3.83–6.20) ملجم / جم على التوالي. الاستنتاج: استوفت طريقة قياس كثافة **TLC** لتحديد **EGCG** والكافيين في الشاي الأخضر والشاي الأسود متطلبات التحقق من صحة الانتقائية والخطية والدقة. لكن دقة هذه الطريقة في تحديد **EGCG** في الشاي الأخضر (5.23٪) والشاي الأسود (7.87٪) تجاوزت متطلبات **AOAC**. في حين أن الدقة في تحديد الكافيين تلبي المتطلبات. كان محتوى **EGCG** في الشاي الأخضر أعلى منه في الشاي الأسود ، بينما كان محتوى الكافيين متماثلًا نسبيًا.

الكلمات الدالة: EGCG ،كافيين ،منتج الشاي الأخضر ،منتج الشاي الأسود ،TLC-Densitometry.

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Effect of *Hylocereus polyrhizus* Extract to VEGF and TGF-β1 Level in Acute Wound Healing of Wistar Rats

Takdir Tahir[1]*, Syakib Bakri[2], Ilhamjaya Patellongi[3], Makbul Aman[2], Upik A. Miskad[4], Maryunis Yunis[5], and Saldy Yusuf[1]

[1]Master of Nursing Science Program, Faculty of Medicine, Hasanuddin University
[2]Department of Internal Medicine, Hasanuddin University Teaching Hospital
[3]Department of Physiology, Faculty of Medicine, Hasanuddin University
[4]Department of Pathology Anatomy, Faculty of Medicine, Hasanuddin University
[5]School of Nursing Moeslim University, Makassar
**Corresponding author.E-mail: takdirtahir@unhas.ac.id*

ABSTRACT

This study aims to identify the effect of *Hylocereus polyrhizus* on the level of VEGFs and TGF-β1 in acute Wistar rat wounds. Male Albino Wistar rats (n = 54) whose (250-350 g) were divided into 3 groups (negative control, positive control, and *Hylocereus polyrhizus*). The animals were wounded at right and left dorsal back with an 8 mm punch biopsy. *Hylocereus polyrhizus* 7.5% topical cream was applied on the wound surface using a cotton bud. The VEGF and TGF-β1 were evaluated with ELISA on the 3rd, 7th, and 14th days. Analyzed using independent t-test and paired t-test (SPSS 21, Chicago Inc.). The *Hylocereus polyrhizus* group had higher level of VEGF and TGF-β1 compared to positive control (p = 0.011; r = -0.584) and (p = 0.000; r = 0.888), respectively, on the 14th day. It was found that there was an upward trend between the TGFβ-1 level and the tissue granulation's score (*p*: 0.051 and r: -0.466) as well as TGFβ-1 levels with epithelialization score (*p:* 0.001 and r: 0.708) in the *Hylocereus polyrhizus* topical group. This study has shown that *Hylocereus polyrhizus* can accelerate wound healing during both the inflammation and proliferation phase.

Keywords: *Hylocereus polyrhizus*, wound healing, VEGF, TGF-β1.

1. INTRODUCTION

There is an increase in the prevalence of acute wounds. The West Wound Prevalence Survey reported that the prevalence of acute wounds is 49%, pressure ulcers are 9%, abraded wounds are 17%, limb injuries are 4%, burns are 1%, malignancy is 1% and other cuts is 9%[1,2]. A study from the United Kingdom (UK) reported a higher prevalence of acute wounds (303 from 826 peoples) involving various forms of a traumatic wound[2]. In Indonesia, the prevalence of acute wounds is higher (64.3%) versus chronic wounds in the home care setting[3]. This burden prevalence of acute wounds is an emerging issue that requires new treatments.

Recently, there has been a renewed interest in complementary therapy. Although various approaches and attempts have been made in clinics to treat acute wounds including debridement, modern wound care dressings, and arterial reconstruction, these approaches are less effective at healing[4]. Meanwhile, empirical experience has proved that the traditional treatment in wound healing that is often applied includes applying a sap or herbs as an anti-bacterial and to prevent bleeding[5,6]. In Indonesia, putting honey on wounds accelerates acute wound healing[7]. Therefore, complementary therapy may have a potential

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role in the wound healing process.

Red dragon fruit is a common complementary therapy in Asia. Red dragon fruit (*Hylocereus polyrhizus*) contains a variety of active substances that can be used in both therapeutic approaches in cardiovascular disease and degenerative diseases[8]. It has many flavonoids, polyphenols, and antioxidants[9]. It also contains β-amyrin (23,3%), ϰ-sitosterol (19,3%), and octadecane (9,2%)[10]. To date, studies investigating *Hylocereus polyrhizus* in the wound care setting are rare. Thus, the purpose of this study was to investigate the potential of *Hylocereus polyrhizus* topical cream to accelerate the healing process of acute wounds in Wistar rats.

MATERIALS AND METHODS

Preparation of red dragon fruit extract

Red dragon fruit was from plantations near Samarinda, East Kalimantan, Indonesia. It is extracted using methanol, and the percentage of inhibition of free radicals (DPPH) was tested. The anti-free radical activity of the red dragon fruit and vitamin C was extracted as much as 314.69 ppm and 3.28 ppm of IC_{50} respectively. Every gram of red dragon fruit contains high levels of polyphenols 1.062 GAE and 8.3 mg of flavonoid content. A topical cream was made with 7.5% of concentration.

Experimental animals

This was an experimental study using a post-test design with a control group design. The samples were healthy male albino Wistar rats (250-350 gr) from the animal laboratory of the Faculty of Veterinary Medicine, Gadjah Mada University. Samples were divided into 3 groups consisting of 18 rats in each group. All groups were treated using primary topical therapy. In Group I (control), rubbing petroleum jelly was applied. Group II (established drug) used Bioplasenton®. Group III used *Hylocereus polyrhizus* as a topical therapy at 7.5% *Hylocereus polyrhizus*. Later, Fixomull® transparent film (BSN Medical) was applied s the secondary dressing. Wounds were then treated and sacrificed on the 3rd, 7th, and 14th days. This study protocol refers to The Council for International Organizations of Medical Sciences (CIOMS) consensus, and this study was approved by the ethics committee of Hasanuddin University (NO. 1684/H4.8.4.5.31/PP36-KOMETIK/2015).

Wounded procedure

Fur from the excision area was removed using cream bleach (Veet®, Reckitt Benckiser) and disinfected with 0.5% of chlorhexidine in 70% alcohol under isoflurane anesthesia (0.01 µg/kg – 0.05 µg/kg)[11]. A circle excision was made in the back area until the fascia profunda with an 8-mm punch biopsy[12]. The diameter of the wound surface area of the excision of all groups was measured using a paper ruler (Figure I).

Histological evaluation of wound healing

Skin tissues were fixed in formalin normal buffer solution 10%, dehydrated in alcohol of various concentrations (70%, 80%, 90%, and absolute alcohol I and II), cleared with xylol, and disembedded in paraffin[13]. The tissue was added to liquid paraffin and was allowed to harden. The tissue was cut with a microtome to five microns. The sample was rehydrated and stained with hematoxylin-eosin (HE). The thickness of the granulation tissue (proliferation) and connective tissue area was measured using a micrometer video JVC Japan with four-fold magnification[14]. Evaluation of wound healing was assessed by scoring the epithelialization and the thickness of granulation tissue[15]. The epithelialization indicator used a semi-quantitative evaluation of histological sections[16], where score 1 = displacement cells (<50%), 2 = transfer of cells (≥50%), 3 = closure of the entire excision, 4 = closure of the entire excision score + keratinization. Meanwhile, the thickness of granulation tissue used a histological score[17], where score 1 = slight, 2 = moderate, and 3 = thick.

VEGF and TGF-β1 measurement

Each group was measured with a biomarker consisting of VEGF and TGF-β1. The VEGF measurements used the ELISA techniques with serum from scar tissue excisions. The VEGF measurement used a mouse VEGF-A enzyme-

linked immunosorbent assay (ELISA; p/n BMS619/2/BMS619/2 TEN). The TGF-β1 mouse ELISA TGF-Platinum kit was p/n MBS608/4/MBS608/4/4 TEN from Affymetrix eBioscience (Vienna, Austria). Examination of VEGF and TGF-β1 was conducted at the Education Laboratory, Hasanuddin University Hospital.

Statistical analysis

Data were reported as the mean and ± SD with a significant level were set up at $p < 0.05$. Spearman correlation proved the correlation between the levels of VEGF, TGF-β1, and length of treatment. Data were analyzed using SPSS software (version 21; SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

DPPH of *Hylocereus polyrhizus*

The IC_{50} of anti-free radical activity and vitamin C of the *Hylocereus polyrhizus* obtained 314.69 ppm and 3.28 ppm (Table I). The results showed that each gram of the red dragon fruit contains about 8.3 mg flavonoid levels and 10.62 mg GAE of total phenol content (Table II).

Table I

The percentage inhibition of DPPH free radical by *Hylocereus polyrhizus* and IC_{50} values.

Replication	Concentration (ppm)	Absorbant	% Retardation	IC_{50} (ppm)
Hylocereus polyrhizus	100	0.635	28.774	314.69
	200	0.589	33.968	
	300	0.485	45.553	
	400	0.382	57.100	
	500	0.320	64.088	
Vitamin C	1.0	0.720	17.676	3.28
	1.4	0.683	21.943	
	1.8	0.565	35.352	
	2.6	0.511	41.600	
	3.4	0.429	50.933	

Table II

Average levels of polyphenols and flavonoids in *Hylocereus polyrhizus*

Sample	Repetition	Absorbant	Polyphenols Level (%)	Mean of Polyphenols Level
Hylocereus polyrhizus	1	0.24	1.090	1.062
	2	0.233	1.041	
	3	0.236	1.059	
Sample	Repetition	Absorbant	Flavonoids Level (%)	Mean of Flavonoids Level
Hylocereus polyrhizus	1	0.074	0.830	0.830
	2	0.074	0.830	
	3	0.074	0.830	

VEGF level on wound healing

The Spearman correlation test evaluated the correlation between the VEGF levels based on the length of treatment. The VEGF levels of *Hylocereus polyrhizus* group increased

between day 3 to day 7 after treatment: 169.17±107.54; p = 0.011; r = -0.702. This declined between the 7th and 14th day (60.34±33.70), but this was not significant (p = 1.000; r = 0.000). In addition, VEGF declined from the 3rd day to the 14th day (46.47±31.26; p = 0.007; r = -0.727) indicating a correlation between VEGF levels with the duration of treatment during the first week. On the contrary, VEGF levels declined in the second week (7th to 14th day) of treatment in the Bioplasenton group and increased after treatment (98.23±66.73; p = 0.015; r = -0.681). Meanwhile, VEGF significantly declined between the 3rd day (107.45±55.87) to the 14th day (27.86 ± 16.49) (p = 0.000; r = -0.874) (Table III).

Table III

Correlation between VEGF levels based on length of Treatment

Group	3rd Day		7th Day 7		14th Day		Correlation		
	Mean	SD	Mean	SD	Mean	SD	3rd-7th day	7th-14th day	3rd-14th day
Control	107.45	55.87	80.49	55.12	27.86	16.49	-0.097	0.389	-0.874***
Drug-Established	55.19	31.61	98,.23	66.73	34.55	21.41	0.438	-0.681***	-0.267
Hylocereus polyrhizus	169.17	107.54	60.34	33.70	46.47	31.26	-0.702**	0.000	-0.727***

*Correlation between VEGF levels based on length of treatment at three different groups were analyzed using Spearman correlation (*p < 0.05, **p <0.01, and ***p <0.001).*

The VEGF levels on day 3 were higher in the *Hylocereus polyrhizus* topical group (169.17 µg/g ± 107.54 µg/g) as well as on day 14 (46.47 µg/g ± 31.26 µg/g) compared to the Bioplasenton and control groups (27.86 µg/g ± 16.49 µg/g). VEGF stimulates angiogenesis, affects epidermal wound closure and repair, and improves granulation tissue and wound repair quality[18]. VEGF effectively accelerates wound closure that is stimulated by angiogenesis, epithelialization, and collagen deposition[19].

This study shows that in macro, wound closure is better in the *Hylocereus polyrhizus* topical group than the control and Bioplasenton group (Figure II adnd Figure III) due to the high levels of VEGF at each stage of wound healing. VEGF illustrates the development of wound healing. During the proliferation phase, VEGF appears approximately 3-7 days after injury in which the capillary growth and differentiation are highest. During this period, VEGF induces the early stages of angiogenesis including dilation, permeability, migration, and proliferation[20].

The mean VEGF levels were higher in the *Hylocereus polyrhizus* topical group (108.84 µg/g) on day 3-7 versus the Bioplasenton group (-43.03 µg/g) and the control group (26.96 µg/g). Moreover, on day 3-14, the mean difference in VEGF was higher in the *Hylocereus polyrhizus* topical group (122.70 µg/g) compared to the Bioplasenton (20.64 µg/g) and control group (79.58 µg/g). This increase in VEGF can predict the occurrence of angiogenesis and better epithelialization process on the wound healing process.

This study is consistent with research concluding that the application of topical VEGF increases epithelialization and increased matrix deposition of collagen and cell proliferation[21]. VEGF also stimulates wound healing through angiogenesis, proliferation, and epithelialization[20]. VEGF is produced by many types of cells that participate in wound healing including endothelial cells, fibroblasts, soft tissue cells, platelets, neutrophils, and macrophages VEGF[22].

VEGF levels on day 3 were higher in the *Hylocereus polyrhizus* topical group (169.17 µg/g ± 107.54 µg/g) as well as on day 14 (46.47 µg/g ± 31.26 µg/g) compared to the Bioplasenton and control group (27.86 µg/g ± 16.49 µg/g). High levels of VEGF are produced during normal wound healing. This produces a strong angiogenic response. VEGF protein levels tend to be low in

individuals who have chronic wounds such as patients with diabetes.

TGF-β1 levels of each group

Levels of TGF-β1 on the 3rd day after treatment were relatively similar across all groups (control group = 143.11±12.80; Bioplasenton group = 143.43±17.69; *Hylocereus polyrhizus* group = 141.98±5.16). There was an increase (p = 0.003; r = 0.775) of TGF-β1 levels on the 7th day after treatment (control group = 167.57±23.37; Bioplasenton group = 148.62±5.93; *Hylocereus polyrhizus* group = 149.59±3.98). While on the day 14th after the treatment, the TGF-β1 level was lower in the control group (153.80±6.55; p = 0.158; r = 0.435), whereas both *Hylocereus polyrhizus* and Bioplasenton group continued to increase, and the trend was nearly identical (178.59±36.62 and 157.53± 11.46 µg/g).

The Spearman correlation test from the control group showed that there was no correlation of time of the treatment (day 3, day 14) with high levels of TGF-β1 (p = 0.158, r = 0.435). The same thing also happened in the Bioplasenton group (p = 0.215, r = 0.386). Otherwise, the result in the *Hylocereus polyrhizus* group (p = 0.000. r = 0.872) implies a correlation of treatment time (day 3 or day 14) with high levels of TGF-β1. This indicated the tendency of TGF-β1 to increase over time during *Hylocereus polyrhizus*-enhanced wound healing (Table IV).

Table IV

Correlation between TGFβ-1 levels based on Length of Treatment

Group	3rd Day		7th Day		14th Day		Correlation		
	Mean	SD	Mean	SD	Mean	SD	3rd-7th day	7th-14th day	3rd-14th day
Control	143.11	12.80	167.57	23.37	153.80	6.55	0.676**	-0.314	0.435
Drug-Established	143.43	17.69	148.62	5.93	157.53	11.46	0.048	0.459	0.386
Hylocereus polyrhizus	141.98	5.16	149.59	3.98	178.59	36.62	0.755**	0.775**	0.872***

*Correlation between TGFβ-1 levels based on length of treatment at three different groups were analyzed using Spearman correlation (*p < 0.05, **p <0.01, and ***p <0.001).*

Correlation of TGF β-1, granulation score, and epithelialization score

Spearman correlation testing between the TGFβ-1 level with the tissue granulation score showed a correlation in the *Hylocereus polyrhizus* topical group that there is a tendency to improves tissue granulation (p: 0.051 and r: -0.466); the control group and the Bioplasenton group showed no correlation between the TGFβ-1 level with tissue granulation's score (p: 0.498 and r: 0.171) and (p: 0.890 and r: 0.035), respectively.

Another research related use of topical concentration red dragon fruit extract 7.5% proved better in repairing the granulation tissue and epithelialization tissue in the Wistar Non-DM group compared with the DM Wistar, so red dragon fruit extract potential to be used as a therapy in wound healing[23].

In the epithelialization score, the Spearman correlation testing between the TGFβ-1 level with epithelialization shows that there is a correlation with *Hylocereus polyrhizus* topical group (p: 0.001 and r: 0.708), while in the control group and Bioplasenton group, we found no correlation between the TGFβ-1 level with the epithelialization score (p: 0.075 and r: 0.429; and p: 0.651 r: 0.127), respectively (Table V).

Table V
The correlation between TGF β-1 and granulation, TGF β-1 and epithelialization based on different groups.

Group	TGF β-1 and Granulation		TGF β-1 and Epithelialization	
	r*	*p*	r*	*p*
Control	0.171	0.498	0.127	0.615
Bioplasenton	0.035	0.890	0.429	0.075
Hylocereus polyrhizus	-0.466	0.051	0.708	0.001

All of data were analyzed using Spearman correlation.

TGF-β1 level on wound healing

The results showed that TGF-β1 undergoes variations in the increased value in each group, but it increases the TGF-β1 on day 3 (inflammatory phase). This does not show a significant difference. The average levels of TGF-β1 increased in the Bioplasenton group (143.43 µg/g). This was slightly lower than the *Hylocereus polyrhizus* topical group (141.98 µg/g) compared to the control group (143.11 µg/g). On the 14th day, the average levels of TGF-β1 decreased significantly in the control group (6.55 µg/g) and the Bioplasenton group (11.46 µg/g) versus the *Hylocereus polyrhizus* topical group (36.62 µg/g).

The presence of TGF-β in the granulation tissues is important for efficient wound healing because TGF-β1 stimulates angiogenesis, fibroblast proliferation, differentiation of myofibroblasts, and matrix deposition. Moreover, TGF-β1 also plays an essential role in the three phases of wound healing: inflammation, proliferation, and maturation. Besides, hemostasis can be defined as a blockage of bleeding after injury, and it is involved in vasoconstriction, platelet collection, and blood coagulation[24].

The TGF-β1 increases the angiogenic properties of the endothelial source cells to facilitate blood supply to the injured area and stimulate fibroblast contraction for wound closure[25]. *Hylocereus polyrhizus* can increase the formation of fibroblast tissue. TheTGF-β1 is higher in *Hylocereus polyrhizus* than the Bioplasenton group and the control group. The results show that the *Hylocereus polyrhizus* topical group has a *p*-value = 0.000 implying that there is a correlation to treatment time (day 3, day 14) with high levels of TGF-β1 (r = 0.872).

Hylocereus polyrhizus also has a good effect on wound healing during the granulation phase. The results indicate that the granulation score of the *Hylocereus polyrhizus* topical group categories of thickness was 50% on day 3, 33.3% on day 7, and 0% on day 14. A Kruskal-Wallis statistical test indicated that $p = 0.026$ which means that there are differences in the granulation score of the *Hylocereus polyrhizus* topical group based on time (day 3, day 7, day 14).

In the maturation phase of wound healing, *Hylocereus polyrhizus* can nicely epithelialize. The wound closure on day 14 in the *Hylocereus polyrhizus* group was keratinized in one of the rats. The Kruskal-Wallis statistical test showed $p = 0.002$, which means that there is a difference in the epithelialization score of *the Hylocereus polyrhizus* topical group as a function of time (day 3, day 7, day 14).

Several studies have been conducted using similar active substances (polyphenolics) with the same content as dragon fruit in the treatment of wound healing in rats with induced allowance[26]. Similar observations have also been done on the effect of dragon fruit extract as a topical cream for granulation tissue. This supports collagen growth because it contains hexosamine that can accelerate the wound healing process[27].

CONCLUSION

Hylocereus polyrhizus topical (7.5%) increases VEGF

levels in the inflammatory phase and increases the levels of TGF-β1 in the proliferative phase. The level of TGF-β1 have a tendency improves tissue granulation and epithelialization. Topical *Hylocereus polyrhizus* 7.5% has the same potential as Bioplasenton during wound healing.
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تأثيرات مستخلصات فاكهة التنين الحمراء (Hylocerus polyrhizus) على مستوى العامل النموي لبطانة الأوعية الدموية (VEGF) وتحويل العامل النموي بيتا 1 (TGF-β1) فى علاج الجروح الحادة لجرذان ويستار

***[1]تكدير طاهر ، [2]سياكب بكري، [3]الهامجيا باتلونج،[2]مكبول أمان، [4]أوبيك أ .مسكاد، [5]ماريونيس يونس،[1] سلادي يوسف،**

1 برنامج ماجستير علوم التمريض ، كلية الطب ، جامعة حسن الدين

2 قسم الطب الباطني ، مستشفى جامعة حسن الدين التعليمي

3 قسم علم وظائف الأعضاء بكلية الطب جامعة حسن الدين

4 قسم علم التشريح الباثولوجي ، كلية الطب ، جامعة حسن الدين

5 كلية التمريض جامعة مسلم ، ماكاسار

المؤلف المراسل*البريد الإلكتروني takdirtahir@unhas.ac.id

ملخص

هذه الدراسة تهدف إلى تحديد تأثيرات مستخلصات فاكهة التنين الحمراء (Hylocerus polyrhizus) على مستوى العامل النموي لبطانة الأوعية الدموية (VEGF) وتحويل العامل النموي بيتا 1 (TGF-β1) في علاج الجروح الحادة لجرذان ويستار . وكانت مادة ألبينو لجرذان ويستار الذكور (n = 54) تنقسم إلى ثلاثة مجموعات، وهي التحكم السلبي والتحكم الإيجابي و فاكهة التنين الحمراء. أصيبت هذا الجرذان–في هذه التجربة–على الظهر الأيمن والأيسر مع خزعة 8 ملم لكمة. وثم وضع كريم مستخلصات فاكهة التنين الحمراء بقدر 7.5 بالمائة على سطح الجرح باستخدام قطعة قطن. واستخدم الباحث المقايسات المناعية المرتبطة بالإنزيم (ELISA) في اليوم الثالث والسابع والرابع عشر من أجل تقييم العامل النموي لبطانة الأوعية الدموية و تحويل العامل النموي بيتا 1. تم تحليل هذه التجربة باستخدام اختبار t المستقل واختبار t المزدوج (SPSS 21, Chicago Inc.). وكان لمجموعة مستخلصات فاكهة التنين الحمراء أعلى المستويات من العامل النموي لبطانة الأوعية الدموية وتحويل العامل النموي بيتا 1 بمقارنة بالتحكم الإيجابي بقدر (p = 0.011; r = -0.584) و (p = 0.000; r = 0.888) على التوالي في اليوم الرابع عشر. لقد وجدنا ارتباطًا بين مستوى تحويل العامل النموي بيتا 1 ودرجة تحبيب الأنسجة بقدر p (0.051) وr (0.466-) بالإضافة إلى مستويات تحويل العامل النموي بيتا 1 مع درجة الاندمال الظهاري بقدر p (0.001) وr (0.708) في المجموعة الموضعية لفاكهة التنين الحمراء. واستنتج هذا البحث إلى أن مستخلصات فاكهة التنين الحمراء يمكن أن يؤدي إلى سرعة علاج الجروح أثناء مرحلة الالتهاب والانتشار.

الكلمات الدالة: فاكهة التنين الحمراء (Hylocerus polyrhizus) ، العامل النموي لبطانة الأوعية الدموية (VEGF)، تحويل العامل النموي بيتا 1 (TGF-β1) ، علاج الجروح.

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Pre-clinical investigation of analgesic, anti-diarrheal and CNS depressant effect of *Pterocarpus indicus* in Swiss albino mice

Ariful Hossen Rajib[1], Mizanur Rahman[*1], Suman Majumder[1], Fahmida Akter[1], Fahadul Islam[1], Masum Shahriar[2] and Jahir Alam [3]

1Department of Pharmacy, Daffodil International University, Dhaka-1207, Bangladesh.
2Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.
3Department of Pharmacy, Gono Bishwabidyalay, Savar, Dhaka-1344, Bangladesh.
**E-mail: mizanur.ph@diu.edu.bd*

ABSTRACT

Objective: *Pterocarpous indicus* is a plant, which is traditionally used for the treatment of tumours, headaches, fever, constipation, stomach pains and female irregular ministration. The present study was undertaken to evaluate the possible analgesic, anti-diarrheal and neuropharmacological activities of the alcoholic extract of *P. indicus* leaves in mice.

Materials and Methods: Swiss albino mice of either sex weighing 25-30gm were used for the study. The analgesic activity was measured by acetic acid-induced writhing inhibition test. Anti-diarrheal activity was assessed using castor oil-induced diarrhea inhibition test and the neuropharmacological activities were evaluated using hole cross, hole board, and open field tests. The extract was used at 250 and 500mg/kg b.w.

Results: The experimental results obtained indicated that the alcoholic extract significantly inhibited the acetic acid induced writhing in mice models at 250 and 500mg/kg body weight. The extract showed a significant sedative effect in hole cross test at 250 and 500 mg/kg. In hole board test, the extract exhibited significant ($p < 0.001$) anxiolytic-like activity at 500 mg/kg. The extract showed no significant anti-diarrheal effect in experimental animals.

Conclusion: This investigation confirmed that alcoholic extract of *P. indicus* has good analgesic and neuropharmacological property. Further studies are required to elucidate the possible mechanisms and to isolate the compounds from the alcoholic extract of *P. indicus* responsible for these bioactivities.

Keywords: *Pterocarpus indicus*, analgesic, anti-diarrheal, anxiolytic, sedative effect.

1. INTRODUCTION

From the very ancient time, various medicinal plants have been used as traditional medicines for remedial purpose. These medicinal plants have the ability of producing many biological effects due to containing various interesting chemical constituents [1]. The unique causes of many momentous drugs that are in current use are plants used by traditional people. Some of the pharmaceutical products presently in use were derived from plant sources which include anti-cancer drugs like vinblastine, vincristine and paclitaxel [2]; analgesics, narcotic like morphine [3] as well as anti- malarial drugs like artemisinin and quinine [4]. Still a significant proportion of the population relies on the conventional system of drugs to treat various diseases [5] and according to assessment by the World Health Organization (WHO), around 80 % of the world population immobile relies on medicines derived from plant sources [6]. Bangladesh is a worthy source of medicinal plants and there are over 500 different medicinal plant species growing in Bangladesh, around 250 of which are used to prepare traditional

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medicines [7]. *Pterocarpus indicus* belongs to the family Fabaceae and is spread throughout the regions of India, Bangladesh and other Southeast Asian countries. In Bangladesh, the plant is known by the name of Padauk used as folk medicine to combat tumors [8]. In Philippines, it is well known by the name of narra. It also exhibits different pharmacological properties [9, 10]. A number of the plant's ingredients have been isolated and studied for their bioactivities. Among them are: angolensin, an antifungal component [11], In India a number of medicinal plants and their formulations are widely used for the treatment of various disorders [12]. Therefore, in the contemporary study we aim to investigate whether the ethyl acetate extract of *Pterocarpus indicus* (leaves) has analgesic, anti-diarrheal and CNS depressant activities.

MATERIALS AND METHODS:

Plant collection and extraction

The leaves of *Pterocrpus Indicus* Willd were collected from Sundarban, Bangladesh, during the month of December 2018. After collection, the sample was identified and authenticated by the authorities of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh and a voucher specimen (DACB: 46477) has been deposited for future reference. The leaves were washed with fresh water, shed-dried and the dried leaves were grounded into fine powder. The crude extract was obtained by cold extraction method by taking 400 g powders in 800 ml of 90% methanol with occasional shaking and stirring. The whole mixture then underwent a coarse filtration by apiece of fresh, white cotton material. Then it was filtered through whatman filter paper. The extract was concentrated initially by rotary evaporator at reduced pressure and finally by open air. The yield was found to be 5.5% w/w. freshly prepared extract was used in the experiments.

Phytochemical screening:

Different phytochemical groups such as alkaloids, tannins, gums, glycosides, flavonoids, saponins and steroids were recognized by distinguishing colour change using standard chemical tests. Molisch Test and Fehling's Test were used for carbohydrate existence. Biurets's Test was used for Proteins detection. Flavonoid Test was used for detection of flavonoids. Alkaloids were detected using the Dragendroff's, Mayer's and Hager's test. For identification of tannin potassium dichromate test, ferric chloride, and lead acetate tests were followed. Keller-Kiliani tests were performed to identify glycosides. Frothing Test for saponins existence, Sulphuric acid test was performed for the detection of steroid. Molisch test was performed for detecting the existence of gum in the samples [13].

Experimental animals:

Swiss-albino mice of 5-6 weeks age and, average weight of 20–25 g were used for the experiment. The mice were acquired from Pharmacy Department, Jahangirnagar University, Dhaka, Bangladesh. The animals were acclimatized to laboratory condition for nine days prior to experiments and had free access to standard pellet diet and water ad libitum properly. The research protocol was approved by the institutional animal ethical committee of Jahangirnagar University, savar, Bangladesh Ref No.: BBEC, JU/M2018 (12) 2 dated: 06.07.2019.

Evaluation of analgesic activity:

Acetic acid induced writhing test

Four groups of six mice each were pretreated with the vehicle only (1% Tween 80, 10 ml/kg, i.p), diclofenac-Na (100 mg/kg) and *P. indicus* alcoholic extract (250 mg/kg and 500 mg/kg) respectively. Forty-five minutes later each mouse was injected with 0.7% acetic acid at a dose of 10 mL/kg body weights. The number of writhing responses was recorded for each animal during a subsequent 3 min period after 15 min of the I.P. administration of acetic acid [14,15].

The percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Mean number of wriths by control} - \text{Mean number of wriths by treated group}}{\text{Mean number of wriths by control}} \times 100$$

Evaluation of anti-diarrheal activity:

Castor oil induced diarrheal test

The method defined by Shoba and Thomas was used for carrying out the test [16]. Before testing, animals were separated for diarrheal test by administering 0.5 mL of castor oil orally and those animals that began diarrhea were selected finally for the test. After selection, selected twenty-four mice were divided into four groups, containing six mice in each group. Mice were fasted for 18 h with free access to water before experiment. Animals of the group 1 received the vehicle only (1% Tween 80, 10 ml/kg, i.p), which served as control; group 2 was treated with standard drug (Loperamide 3 mg/kg body weight). Groups 3 and 4 have received different extract doses 250 and 500 mg/kg, respectively. 30 min after administration, all mice received 0.5 mL of castor oil orally to initiate diarrhea and were individually placed in cages on blotting paper. At every hour, the paper was changed. During an observation period of 4h, the number of diarrheal feces was recorded and the percentage of inhibition of defecation was calculated for every group of animals.

Evaluation of CNS activity:

Open Field Test. In this test, 24 mice were arbitrary selected and divided into four different groups termed as control group, standard group, and test groups (I and II). Control group received only 1% (v/v) Tween-80 at a dose of 10 ml/kg body weight, while standard group received diazepam at a dose of 3 mg/kg body weight as oral suspension. The test groups (I and II) were treated with suspension of plant extracts at the oral dose of 250 and 500mg/kg body weight. All doses were fixed orally with the help of sterile feeding. After respective treatment, animals were placed individually in one of the corners of square grids (100 cm × 100 cm × 40cm). The number of squares traveled by the mice was monitored for 3min at 0, 30, 60, 90, and 120 minutes during the observation period. During the experiment silent environment was strongly maintained [17].

Hole Board Test. Mice were divided into 4 groups and each group contained 6 mice with 25-30g in weight. Group I was given 1% Tween-80, Group II was treated with diazepam at 3 mg/kg body weight dose, and Groups III and IV termed as test groups were given alcoholic extract of Pterocarpus indicus (leaves) *at* the doses of 250 and 500mg/kg body weight, respectively. At the beginning of the test, mouse was placed in the edge of the board. The number of head dips into the holes was counted as the measurement for a period of 3 minutes on 0, 30, 60, 90, and 120 minutes for the entire observation period. The experiment was carried out in a sound attenuated room [18].

Hole Cross Test: After respective treatment of afore-mentioned group, mice were placed individually in the darker chamber of the box, segregated by a wall with hole into dark and white chambers. Within 3 minutes, the total number of crosses from one chamber to the other by the mouse of each group was counted as 0, 30, 60, 90, and 120 minutes. The test was conducted in a sound attenuated room [19].

Data Analysis: Statistical analysis for animal experiments was carried out using Independent-Sample T Test using SPSS 11.5 for windows. Data were presented as Mean±SEM. The results obtained were compared with the vehicle control group. p values < 0.05, < 0.01 and < 0.001 were considered to be statistically significant, highly significant and very highly significant respectively.

RESULTS:

Phytochemical Screening of the crude alcoholic extract of *Pterocarpus indicus* (leaves) was subjected for chemical group tests and discovered the presence of reducing tannin, alkaloid, glycoside, saponins, steroid, flavonoid, phenolic, carbohydrate and terpenoid and absence of gums (Table: 1).

Table: 1 phytochemical test results of extract of Pterocarpus indicus.

Tested groups	Ethyl acetate extract of Pterocarpus indicus (leaves)
Tannins	+
Alkaloids	+
Glycosides	+
Saponins	+
Steroids	+
Flavonoids	+
Gums	-
Phenolic	+
Carbohydrate	+
Terpenoid	+

Evaluation of Analgesic Activity: The crude extract exhibited 38.00%, and 49.63% writhing inhibition in mice at oral doses of 250, and 500mg/kg body weights of mice, respectively. On the other hand, the standard drug Diclofenac sodium exhibited inhibition of 67.44% at 10-mg/kg body weight dose (Table 2).

Table 2: The effect of STD, PI 250, PI 500/kg in the acetic acid induced writhing test.

Treatment	Number of writhing's [Mean ± SEM]	% Inhibition
Control	21.50±2.92	00
Standard [Diclofenac Sodium]	7.0±0.52***	67.44
Leaves extract [250mg/kg]	13.33±1.80**	38.00
Leaves extract [500mg/kg]	10.83±0.75***	49.63

Values are presented as Mean ± SEM (n = 6), ***P < 0.001, **P < 0.01, *P < 0.05, which is significant compared with the control group (one-way ANOVA followed by Dunnett' test).

Evaluation of Anti-diarrheal activity

Castor oil induced method

In castor oil induced diarrhea test, the extract showed no significant antidiarrheal effect in mice. The results are shown in Table: 3.

Table 3: The effect of STD, PI 250, PI 500/kg in castrol oil induced anti-diarrheal test.

Group	Total number of feces (Mean±SEM)	% Inhibition of defecation	Total number of Diarrhoeal feces (Mean±SEM)	% Inhibition of diarrhea
Control	8.0 ± 0.63	00	5.20 ± 0.37	00
Standard	4.4 ± 0.81	45	2.60 ± 0.40	50
Leaves extract -I	7.4 ± 0.51	7.5	3.60 ± 0.93	30.1
Leaves extract - II	7.8 ± 2.55	2.5	4.80 ± 1.85	7.6

Data were analyzed by one-way ANOVA following Dunnet's post hoc test. Values are expressed as Mean ± SEM, n = 6 compared to the control group we found no significant effect.

Open Field Method. The crude extracts shown statistically significant reduction in the movements in mice as compared to control. The decrease in the movement was manifested at 2nd observation persistent until 4th observation at every tested dose (250, and 500 mg/kg). Diazepam exhibited similar results but the effect was fairly stronger than the extracts (Table 4).

Table 4: CNS depressant activity test of leaves extracts by open field method

Group	Dose	Number of movement (% of movements inhibition)				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/kg	5±0.70	6.2±0.37	6.2±0.48	6.6±0.40	8.6±0.24
Standard	3 mg/kg	1.6±0.51	1.2±0.37**	1.6±0.40	1.2±0.58	0.2±0.20*
Leaves extract -I	250 mg/kg	0±0	0.25±0.25***	3±3	2.75±2.75	3.5±3.5
Leaves extract - II	500 mg/kg	0.75±0.48	1.25±0.75**	1±0.71	0.75±0.48	1±0.71*

Values are presented as Mean ± SEM (n = 6), ***P < 0.001, **P < 0.01, *P < 0.05, which is significant compared with the control group (one-way ANOVA followed by Dunnett' test).

Hole Cross Method. The crude extracts shown statistically significant reduction of locomotors activity in mice at every tested dose (250 and 500 mg/kg) compared to control. The decrease in the locomotors activity was manifested at 2nd observation persistent until 4th observation. Diazepam (positive control) exhibited similar results but the effect was fairly stronger than the extracts (Table5).

Table 5: CNS depressant activity test of leaves extracts by hole cross method

Group	Dose	Number of Head dips				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/kg	5±0.70	6.2±0.37	6.2±0.48	6.6±0.40	8.6±0.24
Standard	1 mg/kg	1.6±0.51	1.2±0.37***	1.6±0.40	1.2±0.58**	0.2±0.20***
Leaves extract -I	250 mg/kg	4.25±1.25	2.75±1.25	4.25±2.14	2.25±0.25	5.75±1.03
Leaves extract - II	500 mg/kg	7.75±2.56	2.25±0.96**	4±1.47	1.25±0.25*	1.75±0.48***

Values are presented as Mean ± SEM (n = 6), ***P < 0.001, **P < 0.01, *P < 0.05, which is significant compared with the control group (one-way ANOVA followed by Dunnett' test).

Hole Board Method. In the Hole Board Test, the crude extract at each dose showed significant reduction in the number of head dips compared to control, although the effect of diazepam was stronger than that of the results of the crude extracts. The effect was started from 2nd observation of the experiment and lasted to 4th observation (Table 6).

Table 6: CNS depressant activity test of leaves extracts by hole board method

Group	Dose	Number of movement (% of movements inhibition)				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/kg	5±0.70	6.2±0.37	6.2±0.48	6.6±0.40	8.6±0.24
Standard	1 mg/kg	1.6±0.51	1.2±0.37*	1.6±0.40**	1.2±0.58*	0.2±0.20***
Leaves extract -I	250 mg/kg	4±2.70	3±3.46	3.5±3.11	5±2.22	6.5±1.22
Leaves extract - II	500 mg/kg	8.25±1.31	4.5±0.66	2.5±0.29*	3±0.41	3.5±0.65*

Values are presented as Mean ± SEM (n = 6), ***P < 0.001, **P < 0.01, *P < 0.05, which is significant compared with the control group (one-way ANOVA followed by Dunnett' test).

DISCUSSION

In the contemporary study, phytochemical tests had shown the existence of alkaloid, flavonoid, tannin and saponins in extracts of *P. indicus*. Presence of these phytochemical compounds can be correlated to the biological activities of the test extract. The present study also investigated the analgesic, anti-diarrheal, and neuropharmacological properties of *P. indicus* secondary bioactive metabolites.

Analgesic activity of extract was assessed by acetic acid-induced writhing method. Acetic acid causesd increased levels of local endogenous substances; PGE2, PGF2α as well as lipoxygenase derived eicosanoids in the peritoneal fluid that is being responsible for pain sensation [20,21]. Acetic acid-induced writhing test is widely used method for evaluation of peripheral analgesic effect [22,23]. Agents that lowers the number of writhing, demonstrate analgesia by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition. Leaf extract of *P. indicus* markedly reduced the number of abdominal constrictions and stretching of hind limbs induced by the injection of acetic acid in a dose-dependent manner. This effect was comparable to the effect produced by standard drug diclofenac sodium. The writhing response produced after administration of acetic acid is related to sensitization of nociceptive receptors to prostaglandins. It is established that nonsteriodal anti-inflammatory drugs (NSAIDs) such as diclofenac inhibits the synthesis of prostaglandin; which increases the sensitivity of nociceptor and perception of pain [24].

Therefore the result of the acetic acid-induced writhing model mice suggests that the extract may inhibit the writhing via inhibition of prostaglandin synthesis. [25]. Previous study [26] stated that *P. indicus* stem bark extract also showed significant effect in acetic acid induced writhing test.

It is well established that various flavonoids, alkaloids, steroids are involved in analgesic activity [27]. In the phytochemical group tests of leaf extract, some major phytochemicals namely; alkaloids, tannins, saponins and flavonoids were identified. It is supposed that these polyphenolic compounds may be responsible for analgesic activity of the leaf extract.

Diarrhea can be defined as the abnormally frequent excretion of feces of low consistency, which may be due to a disturbance in the transport of water and electrolytes in the intestines. Castor oil causes diarrhea due to its active metabolite, ricilonic acid that stimulates the release of endogenous prostaglandins and peristaltic activity in the small intestine, leading to the changes in the electrolyte permeability of the intestinal mucosa [28,29]. The results of our study show that there was no significant reduction in the incident and severity of diarrhea with the crude alcoholic extract of *P. indicus* (leaves) in experimental animals.

The Open Field test is one of the most widely used test in animal behavioral studies. The Opening Board Test (HBT) is a trial strategy used in scientific research to quantify tension, stress, neophilia, and emotionality in creatures. Due to its

capacity to quantify numerous practices, it is a prevalent test in conduct pharmacology yet the outcomes are dubious. Also, the approval of nervousness was completed by estimating outer signs, through Hole Cross tests. There are a few reports which exhibited that the alkaloids, glycosides, and flavonoids rich plant extracts possess sedative, anxiolytic, and antiepileptic properties intervened through their proclivity with benzodiazepine site of gabaergic complex framework or are immediate or aberrant modulator delicate his receptor's increases in GABA activity in the brain producing drowsiness and facilitating or maintaining sleep [30-34]. Therefore it appears that the above-mentioned phytochemicals present in the *P. indicus* leaves extract may contribute at least to a limited extent to the calming and sleep inducing impacts on the CNS. We started our examination to assess CNS wretchedness impacts of *P. indicus* departs separate by account unconstrained locomotors action of mice in hole Cross and Open Field tests. Our outcome showed that the oral administrtion of test extract at the dosages 250, and 500mg/kg caused a marked reduction in number of hole crossed and laziness to new condition which was reverse for CNS stimulating agent. All tried portions delivered critical restraint of motion.

The hole board test is useful for demonstrating anxiety in animals. This test is entrenched as a way to examine potential anxiolytic and narcotic impacts of any operators by watching the exploratory conduct in rodents. Head-plunging behavior of the creatures is straightforwardly identified with their passionate state [35]. In light of this perception, it was proposed that the - anxiolytic like state may be reflected by an increase in head –dipping behaviors [35, 36] while an abatement in the quantity of head plunges was seen as corresponded with the depressant impact [37,38]. Our outcomes uncovered that the alcoholic concentrate of *P. indicus* caused a portion subordinate decrease in head- dipping reaction in mice from second perception of the experiment and lasted 04th observation, suggesting that the concentrate has narcotic action.

CONCLUSION

The conclusions of the present study deliver convincing evidence that the extract of *Pterocarpus indicus* leaves holds significant antidepressant and analgesic activity but has no significant antidiarrheal effect. The extract depresses the CNS and possesses significant sedative activity in mice. Thus, these findings provide new data on the activity of the plant, which may serve as lead in drug discovery from natural sources. However, further biochemical and pharmacological studies are required to separate the bioactive compounds and clarify the exact mechanisms responsible for the observed pharmacological activities of this plant.

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دراسه ما قبل سريريه للتأثير المسكن و المضاد للإسهال و المثبط للجهاز العصبي المركزي للمستخلص الكحولي لنبات ال Pterocarpous Indicia علي الفئران

محمد ميزان الرحمن[1]، أريفول حسين رجب[1]، سومان ماجومدير[1]، فهميدا أكتر[1]،
فهد الاسلام[1]، معصوم شهريار[2]، محمد جاهر علم [2]

1 جامعة دافوديل الدولية ، بنغلاديش

2جامعة جهانجيرناغار ، بنغلاديش

3جونو بيشوابيديالاي ، بنغلاديش

ملخص

الهدف من الدراسه : Pterocarpous indicus هي نبات يستخدم تقليديا لعلاج الاورام و الصداع و الحمى و الامساك و عدم انتظام الدورة الشهريه لدي الإناث. أجريت هذه الدراسه بهدف تقييم التأثيرات المحتمله للمستخلص الكحولي لنبات Pterocarpous indicus كمسكن و مضاد الإسهال و تأثيراته العصبيه الدوائيه علي الفئران.

المواد المستخدمه و الطرق المتبعه: تم إستخدام فئران البينو سويسرية من كلا الجنسين تزن ما بين 25 الى 30 غرام فى هذه الدراسة. تم قياس التأثير المسكن عن طريق اختبار تثبيط التلوي/الألم الناتج عن حقن حمض الخلليك. كما تم قياس النشاط المضاد الإسهال باستخدام اختبار تثبيط الإسهال الناتج عن زيت الخروع. تم تقييم الأنشطة العصبيه الدوائيه باستخدام اختبار عبور الفتحات و اختبار اللوح ذو الثقوب داخل حيز مغلق و في مكان مفتوح. استخدم المستخلص بجرعتي 250 و 500 مللي غرام/ كيلو غرام.

النتائج: أظهرت النتائج التي تم الحصول عليها أن للمستخلص الكحولي لنبات Pterocarpous indicia تأثيرا واضحا على تثبيط التلوي المستحث بحمض الخلليك في الفئران بجرعتي 250 و 500 مللي غرام/ كيلو غرام. كما أظهر المستخلص تأثيرا واضحا كمهدئ للفئران في اختبار عبور الفتحات بجرعتي 250 و 500 مللي غرام/ كيلو غرام. كما أظهر المستخلص نشاطا واضحا شبيه بمزيل للقلق عند جرعه 500 مللي غرام/كيلو غرام في اختبار اللوح ذو الثقوب. لم يظهر المستخلص تأثيرا واضحا كمضاد الإسهال لدى الفئران في هذه التجربه.

الاستنتاج: أكدت هذه الدراسه ان للمستخلص الكحولي لنبات ال Pterocarpous indicus خصائص جيدة كمسكن و خصائص عصبيه دوائية. و بناءا عليه فإن هناك حاجه لمزيد من الدراسات لتوضيح الآليات المحتمله لهذه التأثيرات و لعزل المركبات المسؤله عنها من المستخلص الكحولي..

الكلمات الدالة: مسكن، مضاد الإسهال، مزيل للقلق، تأثير مهدئ، Pterocarpous indicus.

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Anxiety and Depression last one Month after Miscarriage at Jordan University Hospital

Asma sa'd Basha[1], Insaf Abu Ghalyon[2], Malab El-Qudah[3], Yousef Elyyan[4], Mohammed Obeidat[5], Zaina Obeidat[6], Haya Alsalihi[7], Ilham Abu-Khader[8]

1 Associate Professor; obstetrics and gynecology, The University of Jordan, and Jordan University of Hospital, Current Email: bashaasma@yahoo.com

2 Resident in obstetrics and gynecology, Jordan University Hospital. The University of Jordan. Email: insaf_abughalyon@hotmail.com

3 Resident in obstetrics and gynecology, Jordan University Hospital. The University of Jordan. Email: malabalqudah@hotmail.com

4 Resident in obstetrics and gynecology, Jordan University Hospital. The University of Jordan. Email:yousef.elayyan1992@gmail.com

5 medical student, Faculty of medicine, The University of Jordan. Email: obeidat-100@hotmail.com

6 Resident in medicine, Jordan University Hospital. Jordan University. The University of Jordan. Email:obeidatzaina@gmail.com

7 Medical Doctor. hayaradhi@hotmail .com

8 MPH, Eastern Mediterranean Public Health Network. Email:iabukhader@yahoo.com

ABSTRACT

Background: Miscarriage is associated with moderate to high risk of psychological problems. In Arab countries, the percentage of people who seek psychological help is low. The epidemiological mental studies for clinical and community samples are not frequently conducted in Jordan. The purpose is to study the rates of anxiety and depression one month after miscarriage and compare them to rates immediately after miscarriage.

Subjects and Methods: A cross-sectional sample of 200 women between June 2018 and December 2019with early pregnancy loss up to 13 weeks of gestation was studied. Assessment for the severity of anxiety and depression was carried out within 12 hours after miscarriage and one month later, using translated and validated versions of the Generalized Anxiety Disorder-7andthe Patients Health Questionnaire 9.

Results: The mean age of women was 33.1± 6.3years and the age ranged between 19-47 years. The number of miscarriages ranged from 1-12 with a mean of 1.9 ± 1.5. Within 12 hours post evacuation; 19.5% of women had severe anxiety and 22.5% had moderately severe and severe depressive symptoms. One month later; 5.5% had severe anxiety and 7% had moderate to moderately severe depressive symptoms. Severe anxiety was transient in about 72% of women and moderate to severe depression was transient in 69%.

Conclusions: Understanding the type and frequency of emotional reactions to pregnancy loss is important. Screening is advised to target appropriate support to those who need, there-by minimizing psychological morbidity and its societal cost. Increasing medical staff awareness is needed.

Keywords: Miscarriage, Anxiety, Depression.

1. INTRODUCTION

Miscarriage is one of the most common complications during early pregnancy (1), and its management is medically straight forward (2). Women are highly reactive to stress in early pregnancy (3). Early pregnancy loss (EPL) is usually a shocking and traumatic event for women and their families (4). At the time of miscarriage, most women experience a period of intense emotional distress (5) that leads to symptoms of grief such as sadness, yearning, social isolation and guilt(6). EPL is a risk factor

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for mental illness (7). Its impact on a woman's life can erroneously be underestimated (7), as it is a significant source of psychiatric morbidity (4). Untreated anxiety after EPL is associated with an increased risk of developing depression (8), as well as a prior pregnancy loss is a risk factor for developing depression and anxiety during future pregnancies especially during their first trimester(9). Although controversial, the presence of anxiety or depression in a new pregnancy constitutes a risk factor for perinatal complications (4).

During the initial weeks following a loss, symptoms of grief may be impossible to distinguish from depression, and some women may continue to experience depressive symptoms for months (5). The psychological impact of miscarriage is sometimes overlooked (2). Expression of grief and depression may show cultural variations (10).Arab cultural traditions; values and beliefs towards mental illnesses are different from those of Westerners (11). Local studies has shown stigma toward mental illness (12-14). The percentage of Arab people who seek psychological help is much lower than those in Western countries (15). Hence, most studies on the psychological impact of miscarriage have been carried out in Western countries(10).Epidemiological mental studies for both clinical and community samples are not frequently conducted in Jordan (12), also there is paucity of studies conducted on mental illness stigma and professional psychological help-seeking (16).

Hence, we designed this study to look into the rate of anxiety and depression in women attending Jordan university Hospital with the diagnosis of EPL immediately and one month later.

We hope this study will attract the attention of health providers dealing with these women.

Subjects and Methods:

All women admitted to Jordan University Hospital for elective termination of previously diagnosed missed miscarriage or retained products of pregnancy on the basis of ultrasound scan, between June 2018 and December 2019 were eligible for inclusion in the study. Each woman had a structured clinical interview by obstetrics' residents within 12 hours after evacuation and before discharge. Those ladies were told that they will receive another phone call from the same residents one month later. They were asked the same questions to fill the same questionnaires. An informed consent was obtained from all participants; they were informed that their participation is voluntary, and that they are free to withdraw anytime during the interview. Women who declined to participate in the study, women who were discharged before the residents were able to interview them, those with multiple gestation and those with miscarriages where fetal measurements were more than 13 weeks by crown rump length were excluded from the study (they were 604 women).We had 100% response rate to the survey phone calls. Hospital records were reviewed to confirm the histopathology of the outcome. A structured clinical interview was conducted by an obstetrical resident using the translated and validated version of the Generalized Anxiety Disorder -7(GAD 7), to evaluate anxiety state and we use Patient Health questionnaire (PHQ 9) for depression evaluation. Respondents were asked to provide information for their age and obstetrical history. They were asked to rate the frequency of anxiety symptoms after evacuation, on a Likert scale which ranges from 0-3. Each item is scored from 0 -3. The 0 is (not at all sure), 1 for (several days), 2 (more than half the days), 3 (nearly every day). The total scores ranged from 0 (no anxiety symptoms) to 21 (all symptoms occurring daily). A total score of 0-4 represents minimal or no symptoms of anxiety, 5-9 mild, 10-14 moderate and 15-21 severe.

For depression symptoms using PHQ-9 questionnaire; there are 9 items for assessment. Each item is scored from 0 (not at all), to 3 (nearly every day). A total score from 0-4 represents the absence or minimal level of depression, 5-9 mild, 10-14moderate, 15-19 moderately severe and 20-27 for severe depressive symptoms. Both questionnaires were completed during the interview with these women.

The study was approved by the Ethics Committee for Medical Research at the Jordan University Hospital and the University of Jordan. Data were analyzed using SPSS 23. We obtained the frequency and percentage of women suffering from mild, moderate and severe anxiety and depression.

We obtained the frequency and percentage of women suffering from mild, moderate and severe anxiety and depression immediately after miscarriage and one month later.

Results: A total of 200women were interviewed; their characteristics were as follows: their mean age was33.1 ± 6.3years, ranged from (19–47) years. Number of miscarriages ranged from (1-12) with a mean of 1.9 ±1.5; their parity ranged from (0-7) with a mean of 2.3±1.5. Table 1

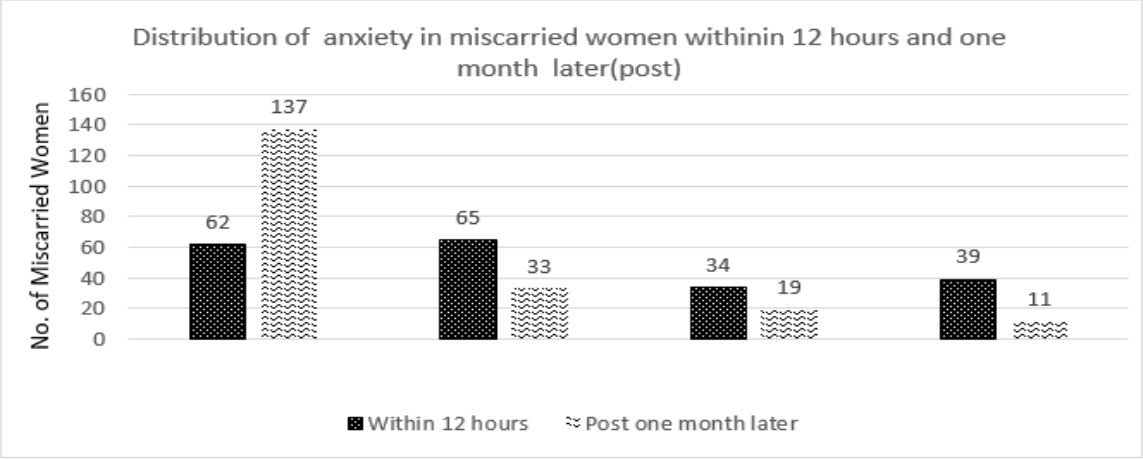
Table 1.Maternal characteristics of women with miscarriage included in the study, Jordan University Hospital, 2020

Maternal characteristics	Women with miscarriage n= 200	Women with ≥ miscarriages n= 92	Women with no previous childbirth n= 27
Age years (mean)	33.1±6.3	34.2± 6.4	29.0±97
Age range (years)	19-47	19-45	19-47
Parity (mean)	2.3 ± 1.5	2.8±1.5	0
Parity range	0-7	0-7	0.0
Number of miscarriages (mean)	2.3±1.5	3.0±1.5	1.6±1.0
Miscarriage range	1-12	2-12	1-4

Our results for anxiety immediately post evacuation showed the following: 62(31.0%) of women had no or minimal symptoms, 65(32.5%) had mild symptoms, 34(17.0%) had moderate symptoms and 39(19.5%) had severe symptoms.Graph1

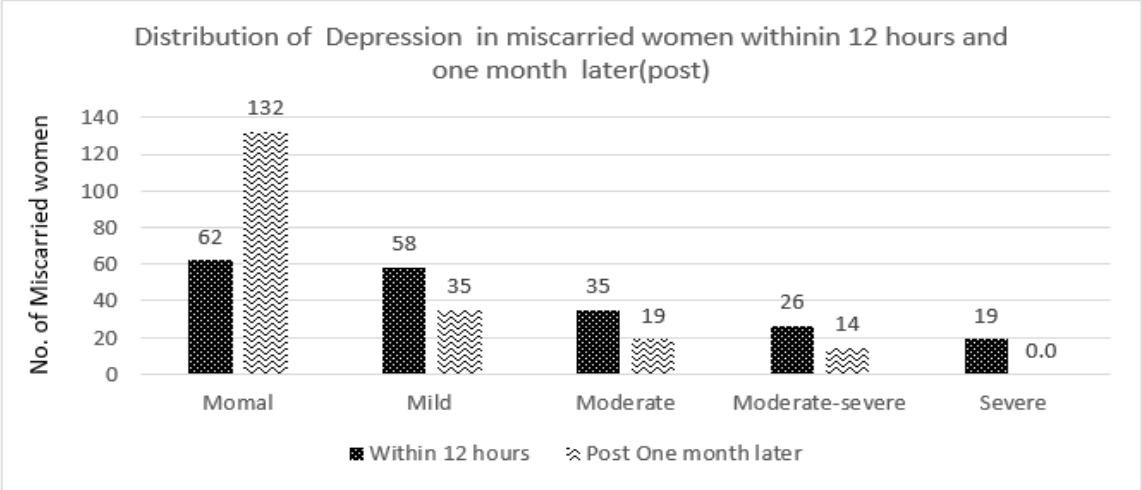
Our results for anxiety one-month post evacuation showed that: 137 (68.5%) of women had no or minimal symptoms, 33 (16.5%) had mild symptoms, 19 (9.5%) had moderate symptoms and 11 (5.5%) had severe symptoms. Graph 1

Graph 1



Regarding depression soon after miscarriage for the total group, there was 62 (31.0%) women with no or minimal symptoms, 58 (29.0%) with mild symptoms, 35(17.5%) had moderate symptoms, 26(13.0%) had moderately severe symptoms, and 19 (9.5%) had severe symptoms. Graph 2, compared to one month later, where 132 (66.0%) showed minimal or no symptoms at all, 35 (17.5%) had mild symptoms, 19 (9.5%) had moderate symptoms, 14 (7.0%) suffer moderately severe depression and no women had severe symptoms. Graph 2

Graph 2



For women with no previous children, 10(37%) had moderate to severe anxiety soon after miscarriage, and 5 (18.5%) stay significantly anxious one month later, compared to 13 (8.1%) who had moderate to severe depressive symptoms soon after EPL and 4(14.8%) still with symptoms one month later.

For women with recurrent miscarriages, we found that 33 (35.8%) of them had moderate to severe anxiety, and 37 (40.3%) had moderate to severe depression soon after EPL. However, one month later 12(13%) suffered from moderate to severe anxiety and 15(16.3%) had moderate to severe depressive.

Discussion:

Miscarriage usually induces an intense period of emotional distress. This reaction tends to improve over the following several months, but some residual psychological concerns remain(1), as some will develop clinically significant anxiety or depression (1,2).There are many studies that focused on anxiety and depression in women soon after EPL and for different periods that follow which ranges from weeks to several months.

Lok IH et al in his longitudinal observational study, showed that (55%) of the miscarrying women scored high immediately after miscarriage on General Health Questionnaire, and 26.8% of the patients scored high on the Beck Depression Inventory (14). However, Prettyman et al, reported clinically important levels of anxiety (41 %) and depression (22 % in the first week following miscarriage (17). Broen et al in their longitudinal study also showed that 10 days after the event, 47.5% of the women who had a miscarriage, had high Impact of Event Scale scores (18).

In another prospective study by Engelhard IM et al, 25% of the women with miscarriages met the criteria for post-traumatic stress disorder at 1 month (19). Jessica Farren et al found at 1 month screen, 32% of women in the EPL group met criteria for moderate-to-severe anxiety,

and the prevalence of moderate and severe depression symptoms 16% (16).Geller et al stated that miscarrying women are at increased risk for anxiety symptoms immediately following miscarriage and this continues until approximately 4 months post-loss (20).

Cumming GP et al found that 28.3% of women had scored at or above the clinical threshold for anxiety and 10% for depression in his prospective study, at 1, 6, and 13 months after miscarriage (5).

Mutiso SK in across-sectional study for depression, found the prevalence of positive depression screen, 34.1% two weeks after a miscarriage (21); however Klier CM et al found these women with significantly increased risk for minor depressive episodes, and majority of these symptoms developed within the first month after miscarrying (22).

Other studies showed the prevalence of depression reduces with time; 26.8 % of patients scored high on the Beck Depression immediately after miscarriage which reduced to 18.4 % at 3 months, 16.4 % at 6 months, and 9.3 % at 1 year after miscarriage (3, 23). In general, anxiety is more marked than depression (24, 25)

In our study, 73 (36.5%) women met the criteria for moderate to severe anxiety, and 80 (53%) of them had moderate to severe depressive symptoms soon after miscarriage, in comparison with 30(15%) of women for moderate to severe anxiety, and 23 (16.5%) of them for moderate to moderately -severe depressive symptoms one month later.

In our study, we noticed that most of women who stay anxious and depressed one month later were nulliparas, had recurrent losses, infertile, or and primigravida with advanced maternal age. Several factors have been identified that can predict which women may experience greater emotional distress, such as one prior miscarriage or recurrent miscarriages, and those with no living children (2, 7, 15, 26).In our study, we noticed that most of women who stay anxious and depressed one month later were nulliparas, those with recurrent losses, infertility and advanced maternal age with IVF.

Mental health care is not integrated within the primary health care system in Jordan (11). Effective screening measures of psychological morbidity in the context of miscarriage have not been established. In 2003, The Scottish Audit of the Management of Early Pregnancy Loss highlighted the need to train healthcare professionals in the identification and management of the emotional and psychological impact of early pregnancy loss (27). The ability of healthcare professionals to detect those most at risk of psychopathology following miscarriage would be greatly enhanced by the availability of a brief screening instrument to be used in a clinical setting by non-mental health professionals (5).Failure to identify those women in need may leave them vulnerable to worsening symptomatology and significant psychosocial impairment (5)..Screening for depression and anxiety (1), and initiating counseling within one week of miscarriage is advised (2), as part of routine care especially when symptoms and signs are present. The primary health care team and hospital staff need to take this into consideration when organizing follow up for women who have had a miscarriage (24).

We hope this study will attract the attention of different medical fields dealing with these women, paving the way for mental status screening after miscarriage and to be followed by proper intervention.

Conclusion:

Understanding the type and frequency of emotional reactions to pregnancy loss is important. Although anxiety and moderate to severe depression were transient in the majority of women (72% and 69%, respectively); still a significant proportion of women will continue to suffer for one month or probably more. Therefore, screening is advised to target appropriate support to those who are in need, there-by minimizing psychological morbidity and its societal cost. Increasing medical staff awareness is needed.

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القلق والاكتئاب يستمر حتى شهر بعد الإجهاض فى مستشفى الجامعة الأردنية

أسماء سعد باشا[1]، انصاف ابوغليون[2]، ملاب القضاة[3]، يوسف عليان[4]، محمد عبيدات[5]، زينة عبيدات[6]، هيا الصالحي[7]، الهام ابو خضرا[8]

1. استاذ مشارك؛ أمراض النساء والتوليد. (الجامعة الأردنية و مستشفى الجامعة الاردنية). bashaasma@yahoo.com
2. طبيب مقيم في أمراض النساء والتوليد, الجامعة الاردنية و مستشفى الجامعة الاردنية.insaf_abughalyon@hotmail.com
3. طبيب مقيم في أمراض النساء والتوليد، الجامعة الاردنية و مستشفى الجامعة الاردنية. malabalqudah@hotmail.com
4. طبيب مقيم في أمراض النساء والتوليد، الجامعة الاردنية و مستشفى الجامعة الاردنية. yousef.elayyan1992@gmail.com
5. : طالب طب ، كلية الطب ، الجامعة الاردنية. الايميل الحالي:obeidat-100@hotmail.com
6. طبيب مقيم في أمراض الباطنية، الجامعة الاردنية و مستشفى الجامعة الاردنية. obeidatzaina@gmail.com
7. طبيب عام. hayaradhi@hotmail.com
8. ماجستير صحة عامة، الشبكة الشرق اوسطية للصحة المجتمعية. iabukhader@yahoo.com

ملخص

الخلفية: يرتبط الإجهاض بخطر متوسط إلى مرتفع من المشاكل النفسية. في الدول العربية نسبة الأشخاص الذين يطلبون المساعدة النفسية منخفضة. لا يتم إجراء الدراسات النفسية الوبائية للعينات السريرية والمجتمعية بشكل متكرر في الأردن. الهدف: هو دراسة معدلات القلق والاكتئاب بعد شهر من الإجهاض ومقارنتها مع المعدلات المقابلة مباشرة بعد الإجهاض. المنهجية: تمت دراسة عينة مستعرضة من 200 امرأة مع فقدان الحمل المبكر حتى 13 أسبوعًا من الحمل. تم تطوع النساء بين يونيو 2018 وديسمبر 2019. تم إجراء تقييم لشدة القلق والاكتئاب في غضون 12 ساعة بعد الإجهاض وبعد شهر واحد ، باستخدام نسخ مترجمة ومصادق عليها من اضطراب القلق العام −7 واستبيان صحة المرضى 9.
النتائج: كان متوسط عمر المرأة المشمولة في هذه الدراسة33،1 ± 6،3 سنة، وتراوحت بين 19−47 سنة. تراوح عدد حالات الإجهاض من 1 إلى 12 بمتوسط 1،9 ± 1،5، وبعد الإجهاض مباشرة (وفي غضون 12 ساعة)، كان لدى19،5٪ من النساء قلق شديد و22،5 ٪ أعراض اكتئاب شديدة وحادة. بعد شهر واح ، كان 5،5 ٪ من النساء يعانون من ا لقلق الشديد و 7 ٪ لديهم أعراض اكتئابية معتدلة إلى معتدلة الشدة. وبعبارة أخرى كان القلق عابرًا في حوالي 72 ٪ من النساء، وكان الاكتئاب المتوسط إلى الشديد عابرًا في 69 ٪ من النساء.
الاستنتاجات: من المهم فهم نوع وتواتر ردود الفعل العاطفية لفقدان الحمل. يُنصح بالفرز لاستهداف الدعم المناسب لأولئك الذين يحتاجون للتقليل من المراضة النفسية وتكلفتها المجتمعية. هناك حاجة لزيادة وعي الطاقم الطبي للحد من معاناة النساء بعد الإجهاض.

الكلمات الدالة: الإجهاض، القلق، الاكتئاب.

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Pharmacy students' knowledge and perception about the implementation of pharmaceutical care services in Jordan

Rana Abu Farha[1], Mohammad Saadeh[2], Tareq L. Mukattash[2*], Anan S. Jarab[2], Mohammad B. Nusair[3], Waleed Al-Qirim[3]

1 Department of Clinical Pharmacy and Therapeutics, Applied Science Private University, Amman, Jordan
2 Department of Clinical Pharmacy, Faculty of pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan.
3 Faculty of Pharmacy, AlZaytoonah University of Jordan, Amman, Jordan
Correspondence to:
Department of Clinical Pharmacy, Faculty of Pharmacy, Jordan University of Science and Technology. P.O.Box 3030, Irbid 22110, Jordan

ABSTRACT

Background: The concept of pharmaceutical care was introduced to the literature by helper and strand before around thirty years, they defined it as "the provision of drug therapy for the purpose of achieving definite outcomes that improve a patient's quality of life". Jordan is still moving slowly toward implementing this service which showed numerous health and economic benefits since its implementation.

Objectives: This study aimed to measure pharmacy the knowledge and perception of pharmacy students approaching the completion of their studies towards pharmaceutical care and their readiness to implement it in their practice after graduation.

Methods: An electronic questionnaire was distributed via the internet to pharmacy and doctor of pharmacy students in public and private universities in Jordan. The questionnaire was composed of 35 question and was divided into three sections. Section one measured knowledge, while section two explored respondents' perceptions of pharmaceutical care. The final section collected respondents' demographic details.

Results: A total of 215 respondent took part in the study. The knowledge score of pharmaceutical care among respondents was 45%, although the vast majority of them stated that they completed at least one module regarding pharmaceutical care during their studies. On the other hand, almost 75% of respondents had a positive perception of pharmaceutical care in Jordan. Results showed statically significant differences among students regarding university type and academic year.

Conclusion: Though respondents completed pharmaceutical care courses during their studies, they had low knowledge regarding the term and its implementation. Combining didactic and experiential education in crucial to build proper practice capacities among future pharmacists.

Keywords: Pharmaceutical care; students; pharmacy; knowledge; perception.

1. INTRODUCTION

The history of pharmacy profession has noticed several turning points, in the past, pharmacists were only responsible for preparing medical products in a small scale, after the manufacturing development the scales got enlarged, in this stage the role of pharmacists changed to compounding, dispensing and labelling preformed medical products (1). However, the most remarkable turning point was the invention of clinical pharmacy by the mid-1960s which adopted a patient oriented practice rather than a product oriented one and allowed pharmacists to interact more with doctors (2).

Despite this drastic change, the introduction of clinical pharmacy concept to the healthcare system was not enough and many outpatients suffered from drug related problems

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that could have been avoidable in a more developed health care system (3). Also, in 1975, Millis report stated that there was a large disparity between the advancement of pharmacotherapy and the level of knowledge regarding the use of therapeutic medications (2). All these factors paved the way for the introduction of the pharmaceutical care concept.

In 1990 the concept of pharmaceutical care was introduced to the scientific literature by helper and strand where it was defined as " the responsible provision of drug therapy for the purpose of achieving definite outcomes that improve a patient's quality of life" (4). Pharmaceutical care service appeared to be beneficial in improving patient adherence and solving drug related problems (5). Also, the application of pharmaceutical care resulted in more favorable scores of health outcomes in many studies (6, 7).

Taking into consideration that pharmacists are easily accessible and require no appointments for consultation (8, 9), this allow them to play a major role in the healthcare system either in treating or preventing diseases as well (10). Currently, more and more countries have authorized pharmacists to prescribe some medications; in the United States for example pharmacists can provide management for dyslipidemia and diabetes for some groups of patients (11).

In Jordan with the introduction of pharmaceutical care modules into the universities pharmacy curriculum and the opening of doctor of pharmacy program and masters of clinical pharmacy program (12), the level of pharmaceutical care knowledge among Jordanian pharmacists was satisfactory but the application of this service was limited (13).

also studies showed that more than half of the Jordanians general public are not familiar with the concept of pharmaceutical care and they perceived that dispensing medications is the major role of the pharmacist (14).

It is important to explore pharmacy students' knowledge about pharmaceutical care and their perceptions regarding it because simply they will be our future pharmacists and to know how to guide our educational policies to optimize our educational outputs. This study is the first of its kind in Jordan targeting this segment, similar studies were conducted in Saudi Arabia and Qatar where students showed very positive attitudes regarding pharmaceutical care (15, 16). Although these studies did not measure students' knowledge of pharmaceutical care instead the researchers were only concerned with the attitudes, thus, the aim of this study is to assess knowledge and perception of undergraduate students about pharmaceutical care in Jordan.

2. Methods

2.1 Study design, subjects and data collection

This is a cross-sectional survey-based study that targeted students in many different public and private universities in Jordan. Data collection was started in May 2019 to September 2019 so it took around four months to reach our sample size of 215 participants from students who study bachelor degree of pharmacy and who study doctor of pharmacy degree as well. We considered the students in the fourth academic year and higher eligible to enter the study.

The survey was electronic and we made sure that the identity of participants kept anonymous. We stated in the survey that filling it would take approximately 15 minutes. Having been given this information, they were asked about their willingness to participate in the survey.

2.2 Questionnaire design

Following an extensive literature review on the pharmaceutical care in Jordan, a questionnaire was proposed and designed to suit our planned scope of study (15-17). The questionnaire was examined by experts in the field of clinical pharmacy to validate it and was piloted on 50 pharmacy students who were excluded from the study results.

The questionnaire consisted of 35 questions divided in three sections: 1) knowledge of pharmacy student of pharmaceutical care, this part consisted of ten true or false statements about pharmaceutical care to evaluate students' knowledge, 2) perceptions of pharmacy students about the

environment, the qualifications and conditions needed to implement pharmaceutical care correctly and this part was mainly in the form of Likert scale questions, 3) demographics to allow us to compare between participants based on their demographic variations.

The knowledge related part consisted of a quiz that was made to evaluate pharmacy students' knowledge about pharmaceutical care. It consisted of 10 statements, some were true, and the others were false. Pharmacy students were awarded one grade if their answers were correct and zero grade if the answer was false. Finally, a total knowledge score out of 10 was calculated for each pharmacy student.

2.3 Ethical approval

The final version of the questionnaire with full study outline were approved by the research on human being committee of the Institutional Review Board (IRB) at Jordan University of Science and Technology (JUST) (Approval No. 435-2019).

2.4 Data analysis

Following data collection, the survey responses were coded and entered into a customized database using the Statistical Package for the Social Sciences (SPSS), Version 21.0 (IBM Corp., Armonk, New York, USA). Descriptive results were presented as means and standard deviations for continuous variables and percentages for qualitative variables. An Independent sample t-test was performed to identify factors significantly affecting knowledge scores. All tests were two-tailed. A P-value of <0.05 was considered statistically significant.

3. Results

In this study, we were able to approach only 215 pharmacy students' who are in their final study years who filled the questionnaire and participated in the study. Of the respondents 81.9% (n= 176) were females while 18.1% (n=39) were males. The average age of respondents was 22.9 years; nearly two-thirds of the participants (n= 142) were enrolled in the doctor of pharmacy program and the around the same percentage of students reported that they are studying in public university with 61.4% of them (n= 123) being in the fifth or sixth academic year. Socio-demographic characteristics of the participants are showed in Table 1.

Table 1. Socio-demographic characteristics of the study participants (n= 215)

Parameter	n (%)	Mean (±SD)
Gender	39 (18.1)	
▪ Male	176 (81.9)	
▪ Female		
Age (years)		22.9 (±2.3)
Academic year		
▪ 4th year or less	83 (38.6)	
▪ 5th or 6th year	132 (61.4)	
Current educational program		
▪ BPharm	75 (34.9)	
▪ PharmD	140 (65.1)	
University classification		
▪ Public University	142 (66.0)	
▪ Private University	73 (34.0)	

Regarding students' knowledge of pharmaceutical care, their knowledge score was 45% in the true or false knowledge evaluating section, which represents unsatisfactory level of knowledge. The lowest three

statements that had correct answers were "Identifying drug therapy problems is a part of the care plan in the pharmaceutical care process", " Any documentation system of drug therapy problems must consist of at least the type of the drug therapy problem and its cause" and " During pharmaceutical care application the pharmacist is responsible of providing the most effective and the cheapest drug" with 3.3% (n=7), 5.6% (n= 13) and 18.6% (n= 40) correct answers, respectively. Table 2 represents the Assessment section of pharmacy students' knowledge of pharmaceutical care.

Table 2. Assessment of pharmacy students' knowledge of pharmaceutical care (n= 215)

Statements	Correct Answer, n (%)
Pharmaceutical care components can be summarized by obtaining drug related needs and identifying drug therapy problems	192 (89.3)
During pharmaceutical care application the pharmacist is responsible of providing the most effective and the cheapest drug	40 (18.6)
Pharmaceutical care plan must be individualized and be obligatory to clinical pharmacists, not all pharmacists	147 (68.4)
Duplication is an example of unnecessary drug therapy	128 (58.6)
The aim of pharmaceutical care is to restore the patient to his normal physiological functioning	41 (19.1)
Taking the patient medical history is a part of the pharmaceutical care assessment process	208 (96.7)
Identifying drug therapy problems is a part of the care plan in the pharmaceutical care process	7 (3.3)
Any documentation system of drug therapy problems must consist of at least the type of the drug therapy problem and its cause	12 (5.6)
Taking too little dose of the correct drug is a problem in the adherence issues	75 (34.9)
Pharmaceutical care is the same as clinical pharmacy	125 (58.1)
Knowledge score (out of 10), mean (SD)	4.5 (1.3)

Despite this low level of knowledge resulted in the questionnaire, 83.3% of respondents (n= 179) reported that they have already started their mandatory training in community pharmacies also 95.4% of the students (n= 205) stated that they were introduced to pharmaceutical care in the university curriculum in at least one module.

The Simple linear regression analysis for risk factors affecting students' knowledge about pharmaceutical care represented statistically significant differences with regard to academic year level and type of university (P-values <0.05) but it failed to show significant differences regarding gender, age and academic program (P-values >0.05). Table 3 represents the results of simple linear regression analysis for risk factors affecting students' knowledge about pharmaceutical care.

Table 3. Evaluation of risk factors affecting students' knowledge about pharmaceutical care (n= 215)

Variables	Students' Knowledge Score	P-value
Gender		
▪ Males	4.3 (±1.5)	0.354
▪ Females	4.6 (±1.2)	
Academic years		
▪ 4th year	4.2 (±1.2)	0.005*
▪ ≥ 5th year	4.7 (±1.3)	
Academic program		
▪ BPharm,	4.7 (±1.2)	0.081
▪ PharmD	4.4 (±1.3)	
University type		
▪ Public	4.7 (±1.3)	0.011*
▪ Private	4.2 (±1.2)	

*Significant at a P-value <0.05

Regarding students' perceptions about pharmaceutical care, 92.1% of the participants (n= 198) stated that they fully support the concept of pharmaceutical care, they also considered pharmaceutical care as a multistep process aiming to improve patients' quality of life whereas 57.2% of them (n= 123) perceived that pharmaceutical care is just an extension to the currently existing pharmacy services. Around 63% of respondents (n= 135) aim to extend pharmacy services beyond dispensing for developing their profession. Only 29.3% (n= 63) agreed that pharmaceutical care services should be paid. Only 21.9% (n= 47) perceived that pharmaceutical care is the doctors' role while nearly 41% (n= 87) thought that doctors and other health professionals will not support a pharmaceutical care role for pharmacists. Addressing teaching of pharmaceutical care, 91.6% (n= 197) considered it important for the students and around half of respondents (n= 121) stated that Jordan universities teach pharmaceutical care clearly. Nearly three-quarters of the participants consider themselves able to identify drug related problems in patients. Table 4 addresses the assessment of pharmacy students' perception about pharmaceutical care.

Additionally, around 41% of participants are satisfied with what they were taught of pharmaceutical care at their universities Also, 19.1% (n= 41) presumed that more than half of the pharmacists in Jordan provide pharmaceutical care at their workplace

Table 4. Assessment of pharmacy students' perception about pharmaceutical care (n= 215)

Statements	Strongly Agreed/Agreed n (%)
Pharmaceutical care requires the use of specialized computer software	150 (69.8)
Pharmaceutical care is just an extension of current pharmacy services	123 (57.2)
In pharmaceutical care the pharmacist identifies and manages a patients existing and potential drug-related problems.	184 (85.6)
Pharmacists require a post-graduate qualification to practice pharmaceutical care in Jordan.	163 (75.8)
Pharmaceutical care involves a defined process, all steps of which must be completed in order to provide this service.	191 (88.8)

Statements	Strongly Agreed/Agreed n (%)
The primary aim of pharmaceutical care is to improve the patients' quality of life.	198 (92.1)
All patients taking medicines require pharmaceutical care	167 (77.7)
The future success of pharmacy will depend on payment for the provision of professional services other than dispensing	135 (62.8)
I fully support the concept of pharmaceutical care	198 (92.1)
Pharmaceutical care is really the doctors' role	47 (21.9)
Pharmaceutical care requires major up-skilling of clinical knowledge	190 (88.4)
Doctors and other health professionals will not support a pharmaceutical care role for pharmacists	87 (40.5)
Jordan's universities teach the principles of pharmaceutical care clearly	121 (56.3)
Teaching pharmaceutical care is important for pharmacy students	197 (91.6)
I think that I have the ability to identify drug related problems in patients	160 (74.4)
I consider pharmaceutical care services should not be offered for free	63 (29.3)

4. Discussion

While a study conducted in Jordan showed that applying pharmaceutical care in patients with type 2 diabetes was correlated with improving HbA1c, fasting blood glucose, lipid profile and patient adherence, but still the concept of pharmaceutical care is still in developing stage (18). This fact can be also deducted from viewing the lack of knowledge about pharmaceutical care from the general public (14). Although a study in Jordan on pharmacists revealed that they had a good understanding level of pharmaceutical care concept while the provision itself for this service remains limited (13). Several problems in the healthcare system and educational system may be concluded from these aforementioned facts, also a study on children revealed unsatisfactory level of knowledge with regard to implementing pharmaceutical care on paediatric patients in final year pharmacy students segment (19). Furthermore, AbuRuz et al. demonstrated the effective role of clinical pharmacist in monitoring medications safety and management of chronic kidney disease complications for hospitalized patients (14). Another study in Jordan found that only 23.1% of pharmacists created a therapeutic plan to be included within the patient's permanent record in their pharmacy, in this study pharmacists showed a desire for practicing pharmaceutical care, however, the application of pharmaceutical care was limited because of lack of pharmacists practice and the rejection of doctors to implement pharmaceutical care (20).

This is the first study conducted on pharmacy students' segment in Jordan to evaluate their level of knowledge about pharmaceutical care and their perceptions with regard to it. The study demonstrates a score of knowledge equals to only 4.5 out of 10, the answers on the ten true or false statements indicates that students were confused in pharmaceutical care steps especially when it comes to identify and resolve drug therapy problems. Nearly 97% of them answered wrongly in a statement classifying "drug therapy problems identification step" in the care plan step in pharmaceutical care.

The answers on the first section also revealed that students in Jordan do not know how to document a drug therapy problem, maybe we can return this to the lack of practical experience on pharmaceutical care among students, although the majority of them reported that they started their 1440 training hours. This fact leads us to question our mandatory training and practices in community pharmacy and investigate the extent of pharmaceutical care application in our community pharmacies.

The level of knowledge of pharmaceutical care appears to be higher in students with higher academic level and those students in public universities rather than private ones. Many explanations may be suggested to justify these findings perhaps the level of knowledge wiring and connection increase while the students proceed in their academic years, also the many factors like the higher competitiveness in public universities may contribute to the increased knowledge score in it.

Regardless of the level of knowledge, students show positive attitudes regarding pharmaceutical care application. The majority of them believed that pharmaceutical care provision is valuable and that the primary goal of pharmaceutical care is improving patients' quality of life. These findings are consistent with other studies conducted in Saudi Arabia and Qatar (15, 16).

More than three-quarters of participants presumed that in order to provide pharmaceutical care the pharmacist must have post-graduate qualification and around 88% of respondents reported that providing pharmaceutical care requires major up-skilling in clinical knowledge, these high percentages for those two questions may indicate that students perceive providing pharmaceutical care as a challenging issue these findings resemble those of the study of pharmaceutical care attitudes in Qatar where 76% of respondents believed that providing pharmaceutical care takes too much time and effort (16)

In our study almost three-quarters of participants perceived themselves able to identify drug related problems in patients while in a study in the United States students rated themselves to have moderate ability in identifying drug therapy problems (21).

Nearly half of the participants only thought that Jordanian universities teach pharmaceutical care principles clearly and around half of the participants indicate that they are not well satisfied with what they had taught in Jordanian universities about pharmaceutical care. These results highlight the increasing need to develop pharmacy curriculum in Jordanian universities to meet student's expectations which focused more on pharmaceutical care topics and maybe implementing training sections during university studies in order to help students practicing pharmaceutical care under specialists' supervision. It is noteworthy here that lack of previous practicing on pharmaceutical care was reported as the top barrier rendering the implementation of pharmaceutical care in in a study conducted on Jordanian pharmacists (13).

This study showed that around 62% of pharmacy students aim to extend their future roles beyond dispensing only and they think that the development of the pharmacy profession will depend on the provision of other pharmaceutical services, perhaps if the statement regarding this point did not include the payment condition the percentage of agreement between students will be higher this assumption could be reasonable when we notice that only 29.3% of the participants agreed or strongly agreed on putting payments on pharmaceutical care services.

One of the major limitations of this study is that findings cannot be generalized and the inherent biases of self-reported questionnaires.

5. Conclusion

Conclusively, pharmacy students showed positive attitudes regarding pharmaceutical care, the participants showed low level of knowledge it despite that the majority of them begun their mandatory pharmacy training, further investigation should be done to figure out the causes of this unsatisfactory level of knowledge and how to solve this problem.

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7. Conflict of interest

The Authors declare that there is no conflict of interest.

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معرفة وتصورات طلاب الصيدلة في الأردن عن خدمات الرعاية الصيدلانية

رنا كمال ابوفرحة[1]، محمد مشير سعادة[2]، طارق لويس مقطش[2]، عنان صادق جراب[2]، محمد نصير[3]، وليد القرم[4]

1 كلية الصيدلة، جامعة العلوم التطبيقية الخاصة

2 كلية الصيدلية، جامعة العلوم و التكنولوجيا الأردنية

3 كلية الصيدلة جامعة اليرموك

4 كلية الصيدلة، جامعة الزيتونة الاردنية

ملخص

مقدمة: تم تقديم مفهوم الرعاية الصيدلانية إلى الأدب العلمي قبل حوالي ثلاثين عامًا، وقد عرف على أنه "توفير العلاج الدوائي لغرض تحقيق نتائج محددة تؤدي إلى تحسين نوعية حياة المريض". في الأردن ما زلنا نمشي ببطء نحو تنفيذ هذه الخدمة التي أظهرت العديد من الفوائد الصحية والاقتصادية منذ ظهورها.

الأهداف: هدفت هذه الدراسة إلى قياس مستوى معرفة طلبة الصيدلة في السنة النهائية بمبادىء الرعاية الصيدلانية وتصوراتهم بشأنها في الأردن. الأساليب: تم توزيع استبيان إلكتروني عبر الإنترنت على طلاب الصيدلة و دكتور الصيدلة في الجامعات العامة والخاصة، وتم تقييم معرفتهم بالرعاية الصيدلانية في القسم الاول من الاستبيان، من خلال عشرة جمل اختيارية تتراوح اجابتها بين الصحيح والخطأ، والقصد من القسم الثاني هو تحديد تصورات الطلبة بخصوص الرعاية الصيدلانية بينما يهدف القسم الثالث الى تحديد التركيبة الديمغرافية للمشاركين.

النتائج: أجاب ما مجموعه 215 طالبًا على الاستبيان، وكانت درجة المعرفة بالرعاية الصديدلانية بين الطلاب 45٪ على الرغم من أن الغالبية العظمى منهم ذكرت أنهم أخذوا مساقا واحدا على الأقل فيما يتعلق بالرعاية الصيدلانية في سنوات دراستهم، بالاضافة الى ذلك أظهر 75٪ من الطلاب تصورات ايجابية مرتبطة بخدمة الرعاية الصيدلانية. أظهرت النتائج وجود فروق ذات دلالة إحصائية بين الطلاب فيما يتعلق بنوع الجامعة والسنة الاكاديمية للطلبة.

الخلاصة: كان مستوى المعرفة غير مرضيًا مما يشير إلى أنه ينبغي للجامعات التركيز أكثر في تدريس مواد الرعاية الصيدلانية فيها وتطبيق تدريب ميداني على مبادىء الرعاية الصيدلانية

الكلمات الدالة: الرعاية الصيدلانية، طلاب الصيدلة، معرفة، الأردن .

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الناشر

الجامعة الأردنية
عمادة البحث العلمي
عمان 11942 الأردن
فاكس: 5300815 6 00962

رقم الإيداع لدى دائرة المكتبة الوطنية
(2008/23.3/د)

المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

تأسست المجلة الأردنية في العلوم الصيدلانية بقرار لجنة البحث العلمي/ وزارة التعليم العالي والبحث العلمي رقم 367/2/10 تاريخ 2007/1/11 بشأن إصدار "**المجلة الأردنية في العلوم الصيدلانية**" ضمن إصدارات المجلات الأردنية الوطنية، وهي مجلة علمية عالمية متخصصة ومحكمة، وتصدر بدعم من صندوق دعم البحث العلمي والجامعة الأردنية تعنى بنشر البحوث العلمية الأصيلة المقدمة إليها للنشر في كافة مجالات العلوم الصيدلانية والعلوم الأخرى المرتبطة بها. وتصدر عن عمادة البحث العلمي وضمان الجودة في الجامعة الأردنية باسم الجامعات الأردنية كافة، خدمة للمتخصصين والباحثين والمهتمين في هذه المجالات من داخل الأردن وخارجه. وهي مجلة تصدر أربع مرات في العام أعتبارا من 2021، ومواعيد صدورها (آذار وحزيران وأيلول وكانون أول) من كل عام.

وباسمي وباسم أعضاء هيئة التحرير نود أن نشكر الزملاء الذين أسهموا بإرسال أبحاثهم إلى مجلتنا وتمكنا من إخراج العدد الأول. ونأمل من جميع الزملاء بإرسال ملاحظاتهم الإيجابية إلينا لنتمكن من النهوض بمجلتكم بالشكل الذي يليق بها.

وهذه دعوة إلى كافة الزملاء لإرسال اسهاماتهم العلمية من الأبحاث الأصيلة إلى عنوان المجلة.

والله ولي التوفيق

رئيس هيئة التحرير
أ.د. إبراهيم العبادي
قسم الصيدلة الحيوية والسريرية
كلية الصيدلة– الجامعة الأردنية
عمان 11942–الأردن