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INTRODUCTION

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

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

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

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 researchers,

After nearly two years of the outbreak of the contagious coronavirus disease (COVID-19), the pandemic is still negatively impacting the public health, education, and global economy. Herein, current advances that can draw attention and speculate on the future directions for COVID-19 are deliberated.

The knowledge regarding the life cycle of the virus revealed several host-based and virus-based targets that can lead the medicinal chemistry and the drug development research toward the rapid discovery of novel drugs for this alarming disease using different strategies like drug repurposing and screening of existing drug databases, or *de novo* synthesis. COVID-19 proteins and enzymes involved in viral infection are potentially druggable targets which could be either structural proteins such as: spike (S) glycoprotein, and nucleocapsid protein, or non-structural proteins such as: proteases, helicase, and RNA-dependent RNA polymerase. Besides, several host-based druggable targets are available for drug discovery such as: ACE2 receptor, trans-membrane serine protease 2, furin, cathepsin L, kinases, and two-pore channel.

Meanwhile, there is an imperative need to find out small-molecule antiviral drugs and vaccines, and since the drug discovery and lead development processes are time-consuming and expensive, bioinformatics, chemo-informatics studies, *in silico* ligand-based and target-based virtual screening, *in vitro* screening, molecular dynamics, and molecular modeling techniques are the handy strategies for the identification of promising lead compounds against putative targets and the design of pan-coronavirus antiviral drugs or multi-target approach to avoid viral mutation. Moreover, the availability of X-ray crystal structures of critical viral proteins will trigger more docking studies.

Jordan Journal of Pharmaceutical Sciences (JJPS) welcomes your new and significant scientific contribution on various topics such as synthetic organic chemistry, characterization, computational chemistry, combinatorial chemistry, rational drug design, molecular modeling, drug discovery, high-throughput screening, understanding mechanism of action, structure-activity relationship, and structure-ADME relationship of bioactive compounds.

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CONTENTS

Evaluating pharmacy students' perceptions of depression and psychotropic medicines in the Hashemite Kingdom of Jordan

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ABSTRACT

Background: Although enhancing of pharmacy students' knowledge and perceptions of mental illness is important for future career development, the pharmacy curricula in Jordan are largely deficient in mental health courses and no study has examined the attitudes of pharmacy students towards mental illness and their knowledge on psychotropic medicines in Jordan. ***Objective:*** To evaluate pharmacy students' knowledge on depression and psychotropic medicines and to assess their attitudes towards providing pharmaceutical care (PPC) to patients with depression. ***Method:*** A cross-sectional emailed survey was conducted on a purposive sample of 200 pharmacy students who were in 4th and 5th level in one Jordanian university. The questionnaire included four sections; demographics, knowledge on depression, attitude towards PPC to patients with depression, and knowledge on psychotropic medicines. Chi-square testing was used to determine significant relationships between demographics and other statements. ***Results***: A total of 134 responses were received (response rate 67%). Most of students believed that patients with depression will not take antidepressants forever (n=104, 77.6%). Less than half (n=60, 44.8%) of students thought that antidepressants do not cause addiction. Eighty-seven students (64.9%) were able to monitor efficacy and adverse effects of antidepressants. Only about a third (n=52, 38.8%) of students knew venlafaxine, and 47.0% of students (n=63) didn't know vortioxetine. Having training courses on psychiatry were significantly associated with knowledge of pharmacy students in psychiatry ($p<0.05$). ***Conclusion***: Despite students expressed positive attitude towards PPC to patients with depression, pharmacy students should improve their knowledge on psychiatric pharmacotherapy. Policy makers should include courses on psychiatric disorders and pharmacotherapy in university curricula.

Keywords: Attitudes , depression, mental illness, pharmacy students, pharmaceutical care, perception.

INTRODUCTION

The positive dimension of mental health is illustrated in the World Health Organization's (WHO) definition as: *"health is not merely the absence of disease or disability but it is a condition of complete well-being whether physical, mental and social well-being".* However, mental illness is defined as *"clinically significant conditions characterized by fluctuating in thinking, emotions and behaviour related with personal distress and/or impairment in function*".[1-2]

Depression is a common mental illness that presents with depressed mood, loss of interest or pleasure, decreased energy, feelings of guilt or low self-worth, disturbed sleep or appetite, and poor concentration. Moreover, depression is a major cause of disability across the world. This disorder is fundamentally interconnected with physical and social functioning and health outcomes.[3-4]

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In Jordan, there is no main mental health authority in the country. The Head of Mental Health Specialty and the Director of the National Center for Mental Health in the Ministry of Health are the main authorities. The National Center for Mental Health (Karama) is the lead agency for the provision of mental health services, treatment and awareness, supervision and training [5]. A majority (three of four) of the mental hospitals are organizationally integrated with mental health outpatient facilities, meaning only that each hospital has its own outpatient clinic. There is only one-day care center for mental health and it is private, and there are no community-based psychiatric inpatient units or community residential facilities [6]. According to WHO (2017), depression was the second most common mental illness after anxiety in Jordan. The total estimated number of people living with depression in Jordan was more than 287 thousand (4.0% of the population)[3].

Notably, the bachelor of pharmacy (BPharm) curricula in the Jordanian universities comprised of 3 main departments: Biopharmaceutics and Clinical Pharmacy, Pharmaceutics and Pharmaceutical Technology, and Medicinal Chemistry and Pharmacognosy. In addition to 1440 hours of practice training in community pharmacies, hospitals, or industry. These curricula focus on over-the-counter drugs (OTC) and chronic physical diseases such as asthma, diabetes, or hypertension. However, these curricula are largely deficient in mental health courses and clinical training.[7-8]

Several studies investigated the attitude of pharmacy students towards PPC to patients generally [9-10]. Other studies investigated the attitude of pharmacy students towards mental illnesses and suggested more training courses on psychotropic medicines and mental health to prepare pharmacy students to PPC to this population [11-12-13-14].

Understanding the attitude of pharmacy students towards PPC to patients with depression and their knowledge on psychotropic medicines are very important to develop the future role of pharmacists in psychiatry [15]. This study will add valuable information to the literature and can be used as a frame of reference for future studies as it is the first study to investigate pharmacy students' perceptions and knowledge of psychiatry in Jordan.

Objective

The purpose of this study was to evaluate pharmacy students' attitude towards PPC to patients with depression and their knowledge on depression and psychotropic medicines.

Method

A cross-sectional emailed survey was sent to all 200 pharmacy students (fourth and fifth year) in one Jordanian university. Those who were in the 1st, 2nd and 3rd year were excluded

because they have not yet studied pharmacotherapy nor undertaken community pharmacy placements.

Ethics approval was obtained from the Institutional Review Board (IRB) at the University of (REDACTED) on 18th of July 2018. The consent to participate was implied by the act of completing and returning the e-survey.

Development of survey instrument

Following an extensive literature review on studies covering the evaluation of pharmacy students' perceptions of mental illness, a draft of an electronic survey was designed in order to cover the areas of our interest in this study. The consent to modify the questionnaire was obtained from Marshall Cates in March 2018[16]. The questionnaire was written in two languages; it was written in the English language because it is the medium of instruction in Jordanian universities and was translated to the Arabic language as Arabic is the first language in Jordan. The translation was validated by the Translation Department at the University followed the standard 'forward-backward' procedure. The final version of the questionnaire was further tested for content validity by experts in the filed who gave their constructive suggestions, positive feedback for the process [17]. To ensure practicality, a pilot study was conducted by administering the survey to 10% of the parent sample (n=20) not included in the full survey [18].The survey was divided into

the following sections: Demographic section gathered information about pharmacy students' characteristics. Section I included questions to establish students' knowledge on depression. Section II measured students' attitude towards PPC to patients with depression. This section included a set of statements for which respondents were asked to indicate their agreement using a 3-point Likert scale. For the purpose of analysis, responses showing more interest or willing to do in the future (agree) as well as for the less interest and willing to do in the future (disagree and neutral) were combined. According to the 3-point Likert Scale, the minimum possible mean for each item was 1.00, and the maximum possible mean for each item was 3.00. The mean for each item was measured. The author judged that the answer was "agree" when the mean range was 1.00-1.66, which indicated to a "positive attitude". On the other hand, when the mean range was 1.67-3.00, the author judged that the answer was "disagree or neutral", which indicated to a "negative attitude". Section III included questions to evaluate students' knowledge on the most commonly psychotropic medicines prescribed in Jordan. Correct answers in the knowledge questions (section I and III) were based on the British National Formulary 2018 (a standard pharmaceutical reference book used in Jordanian pharmacy faculties).

Description of sample

The electronic survey was sent to 200 students studying in the Faculty of Pharmacy in the University of (REDACTED). Only who were in 4th and 5th level were included. Those who were studying in the 1st, 2nd and 3rd year were excluded because they did not study pharmacotherapy courses and they did not have training courses in the simulated model pharmacy or community pharmacies. One month later the survey was resent to the 200 students to increase the response rate.

Sample Size Determination

All the pharmacy students who were in the fourth and fifth year in the Faculty of Pharmacy at the University of (REDACTED) constituted the population of the study. The formula which used to calculate the sample sizes was;

$$n = N/(1 + N(e\hat{}(2\,)\,)\,)$$

A confidence level of 95% was used to calculate the sample size. With a population size of 200 students, the necessary sample size was determined to be 132 students (www.raosoft.com).

Statistical analysis

The data were analysed using IBM SPSS 24. The internal reliability was evaluated by measuring the Cronbach's alpha. The descriptive analysis was performed using frequency/percentage for qualitative variables. Chi-Square testing was used to determine the relationship between the demographics and other statements.

Results

Response and respondent characteristics

A total of 134 completed the survey. The response rate was 67% (134/200). Respondent characteristics are shown in **Table 1**. The majority of students were female (n=81, 60.4%). Around two-thirds of students (n=87, 64.9%) were in the fifth year. Only 38.8% (n=52) had received a training course on psychotropic medicines and 27.6% (n=37) of students had received training on mental illness. More than half of students (n=77, 57.5%) knew someone who had experienced mental illness.

The knowledge of pharmacy students on depression

The majority (n=101, 75.4%) of students thought that depression is a common illness in Jordan and 112 students (83.6%) believed that children are able to experience depression. Only 48.5% (n=65) of the students believed that side effects of antidepressants are not worse than depression, and less than half (n=60, 44.8%) of them thought that antidepressants do not cause addiction. Less than half of students (n=66, 49.2%) believed that people with depression understand the information provided by pharmacists about their medications. The students who think that patients with depression will not take

antidepressants forever were 104 (77.6%) (**Fig 1**). Reliability for this section, as evidenced by Cronbach's alpha, demonstrated relatively high internal consistency (α= 0.71) [19].

The attitude of pharmacy students towards providing pharmaceutical care to patients with depression

In general, 59.0% (n=79) of students were keen to obtain a medication history for patients with depression and to monitor efficacy and adverse effects of antidepressants. There were 84 students (62.7%) who were able to give patients with depression enough time to discuss their medications. Additionally, most students (n=83, 61.9%) were able to talk to patients with depression in a private area about their medications. The students were almost evenly split between able and not able to suggest antidepressants or changes in antidepressants dosages to doctors (**Fig 2**). The internal consistency for pharmacy students' attitude demonstrated high reliability (α= 0.73*)* [19].

The knowledge of pharmacy students on psychotropic medicines

There were 44 students (32.8%) who agree that mood stabilizers are not first line for depression treatment and 71 students (53.0%) answered (I don't know) for this statement. Only 38.8% of students (n=52) knew vortioxetine and venlafaxine and 47% (n=63) didn't know quetiapine. Students were almost evenly split between those who knew anxiolytics, such as alprazolam and diazepam and the students who didn't know the correct answers **(Fig 3)**.

Relationship between pharmacy students' variables

There were no major findings in regard to comparing demographics with students' attitudes. However, there were a number of statistically significant demographics when they were cross-tabulated with the students' knowledge. The age group 24-25 years had a higher percentage of correct answers compared with other groups *(p value* =<0.0001). Women tended to answer correctly 2-3 times higher than men *(p value* =0.002). The students who had a training course and answered correctly were 1.5-2 times more than those who did not have any training course and answered correctly *(p value* =0.001). However, the students who did not have a course answered "I do not know" 2-4 times higher than the students who had a course.

Discussion:

In the present study, we aimed to evaluate pharmacy students' perceptions of depression and their knowledge on psychotropic medicines.

In this study, most of students incorrectly believed that antidepressants cause addiction. This may be because the majority of the students had not received any training course on psychiatry or psychotropic medicines. Since most of the students mistakenly thought that antidepressants cause addiction, they have also incorrectly believed that side effects of antidepressants are worse than depression itself. Traditional pharmacy education, including lectures and tutorials-led by pharmacists, additionally, new models of pharmacy education involving greater participation of students in psychiatric clinics may well be useful to improve students' knowledge on psychiatry pharmacy practice. Previous research suggested that more comprehensive education and training programmes were important to develop the comprehension of pharmacy students about mental illness to meet the needs of mentally ill patients [11-12-13-20]. Most of students in our study believed that patients with depression don't understand information provided by pharmacists about their medications. This answer was reflective of the negative perception towards mentally ill people. This could have made most of students abstain from following up with patients regarding their medications in future. According to the Modified Theory of Planned Behavior (MTPB) [21], knowledge significantly affects attitude, and consequently attitude affects behaviour.

Pharmacy students expressed good, unprejudiced attitude towards PPC to patients with depression. This finding was consistent with several studies [9-10-22];

Alarifi's study showed positive attitudes towards PPC by students in Saudi Arabia [10]. Most students in our study were keen to give patients with depression enough time to discuss their medications and talk to those patients in a private area about their medications. This result was congruent with a previous study in the UK, which indicated that more than half of the students were confident to discuss mental health issues with patients [22]. On the other hand, the students in our study acknowledged that they were not able to suggest antidepressants to doctors. This was consistent with other studies [23-24], which revealed less positive attitudes in decision-making about psychotropic medications. This might be due to students' poor knowledge on psychotropic pharmacotherapy; as more than two-thirds of the students did not have any training course on psychiatric medications. Any negative attitude of pharmacy students towards PPC is considered as a potential barrier towards dealing with mentally ill patients in future.

Expectedly, the majority of students were not sufficiently informed about psychotropics. The lack of mental health pharmacy education could be considered as the major reason behind poor knowledge. This was consistent with Farmer *et al* who revealed that students had poor knowledge regarding psychotropic medications [25]. Pharmacists are frequently consulted on psychotropics within their communities. Medication counselling can improve patient's adherence to medications used for mental illnesses in particular. Hence, if the pharmacy students haven't had appropriate knowledge on psychiatry pharmacotherapy, this could well enhance the stigma towards and miscommunication with the mentally ill patients.

We failed to find a relationship between the level of study and students' attitude towards PPC to patients with depression. Unsurprisingly, having a training course on psychotropic medicines or mental illness was the most significant factor. This means that the students improved their knowledge to some extent on psychiatric pharmacotherapy by attending related courses, which emphasises the need to focus on psychiatric pharmacotherapy courses to increase students' familiarity with mental illness, and thereby boosting their positive attitude towards the mentally ill. Bell and Cate revealed the positive impact of psychiatric clinical rotation on students' attitude towards mental illness [14-26]. Gable *et al.* (2011) argued that advanced training in mental conditions is vital to develop pharmacy students' perceptions and prepare them to provide unbiased patient-centered care [12].

Our study added valuable information to the literature, since this study highlights, for the first time in Jordan, the perception of pharmacy students about depression and psychotropics. Therefore, it can be used as a frame of reference for future studies.

Several limitations of our study must be taken into account. First, the e-survey was sent to students in one particular location. Therefore, the students' attitudes or knowledge may not be representative of all pharmacy students in Jordan. Second, the questionnaire contained questions about specific types of psychotropic medications rather than covering all types of psychotropic medications.

Future Research Recommendations:

Understanding the attitudes of pharmacy students toward people with mental disorders is very important for developing the future role of the pharmacist in supporting people with a mental illness. Larger studies investigating pharmacy students' perceptions on mental illness using a larger sample size at other universities in Jordan should be conducted. Future studies should evaluate specific educational experiences within the undergraduate curriculum using before/after study design. Following up with students during their experiential education clerkships and again after graduation would also be beneficial. Further studies should be conducted to investigate their perceptions of other mental illness such as schizophrenia and obsessive-compulsive disorder.

Conclusion

In general, pharmacy students expressed overall

positive attitudes towards PPC to patients with depression. They, however, had poor knowledge on depression and psychotropic medicines. The results of our study suggested more educational programmes that address and develop pharmacy students' knowledge on psychiatric pharmacotherapy. Policymakers should include courses on mental illness and related medications in university curricula for pharmacy students. New educational strategies may be also required to foster students' attitudes toward people with mental illness.

Acknowledgements

We thank all pharmacy students who participated and provided insight that greatly assisted the research.

Table 1. Demographic variables for students' sample (n = 134)

Characteristic	*n (%)*
How old are you?	
<20	*7 (5.2)*
20-21	*37 (27.6)*
22-23	*43 (32.1)*
24-25	*47 (35.1)*
What's your gender?	
Female	*81 (60.4)*
Male	*53 (39.6)*
Which university year are you in now?	
Fourth year	*47 (35.1)*
Fifth year	*87 (64.9)*
Have you ever visited an institution for patients with mental illness?	
Yes	*19 (14.2)*
No	*115 (85.8)*
Have you ever had a training course on psychiatric medications at a university or another venue?	
Yes	*52 (38.8)*
No	*82 (61.2)*
Have you ever had a training course on mental health conditions at a university or another venue?	
Yes	*37 (27.6)*
No	*97 (72.4)*
Have you ever known someone with a mental illness?	
Yes	*77 (57.5)*
No	*57 (42.5)*

Fig 1 The proportion of students that displayed correct knowledge on depression (n=134)

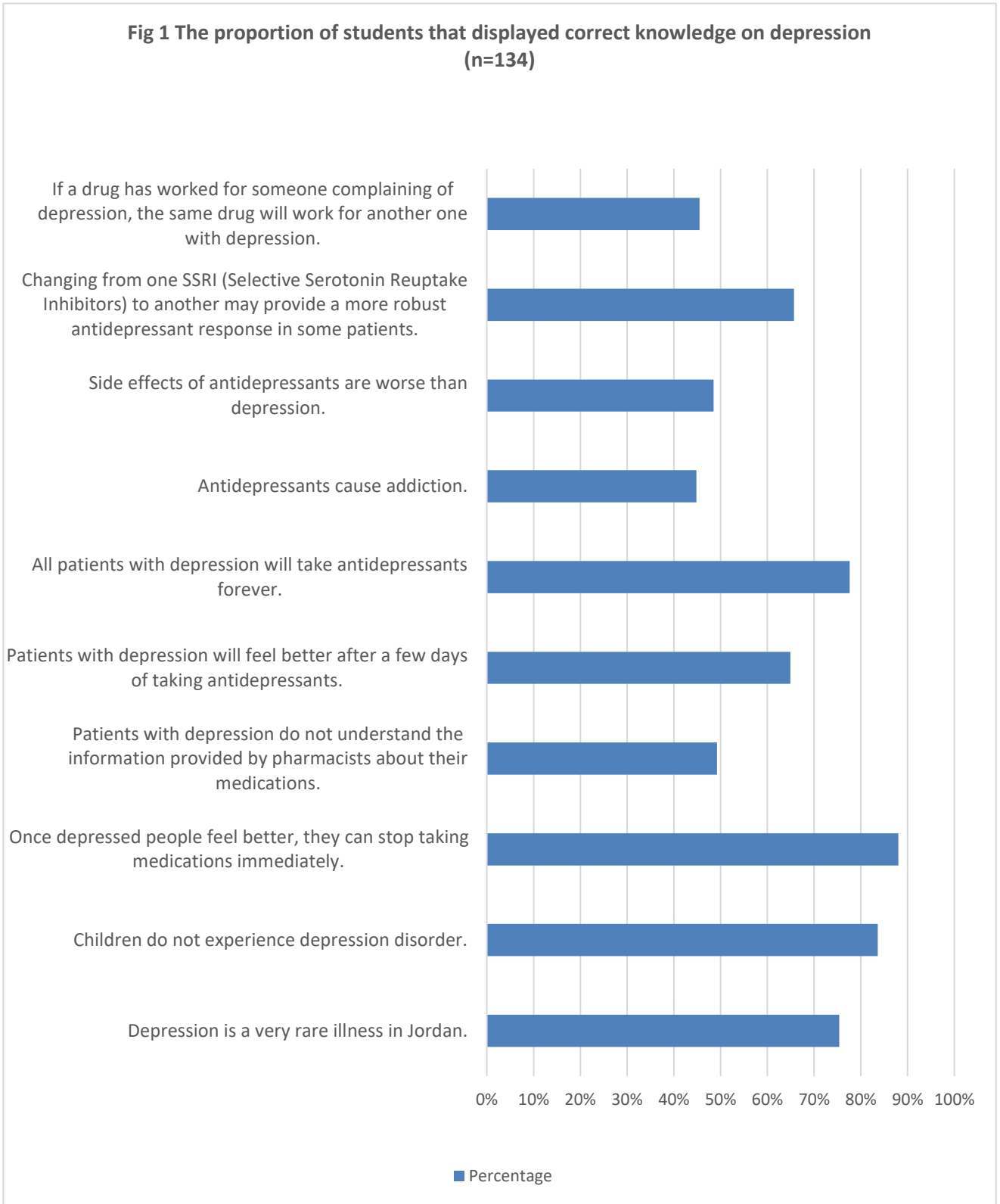


Fig 2 The proportion of students that expressed positive attitudes towards providing pharmaceutical care to patients with depression (n=134)

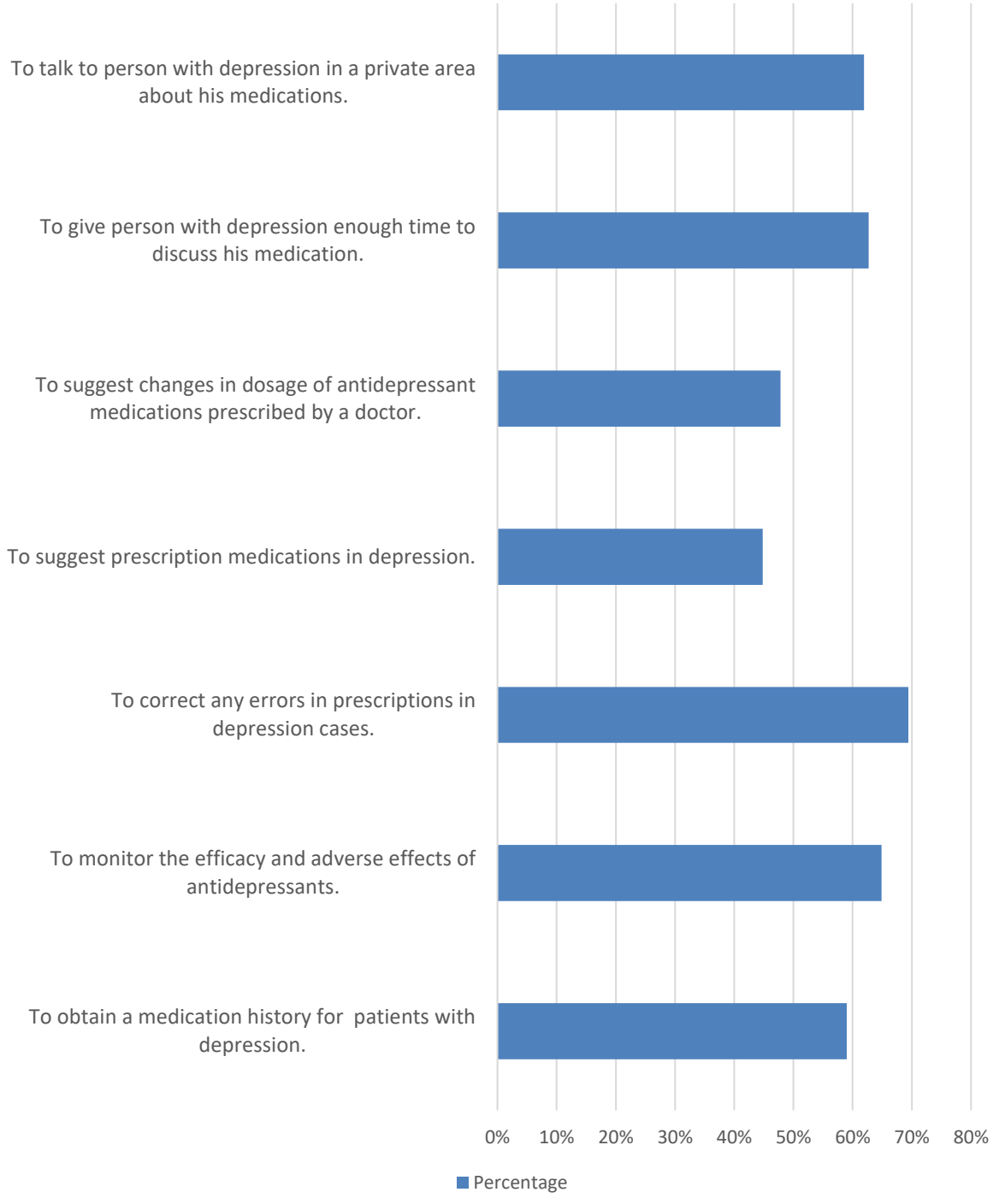
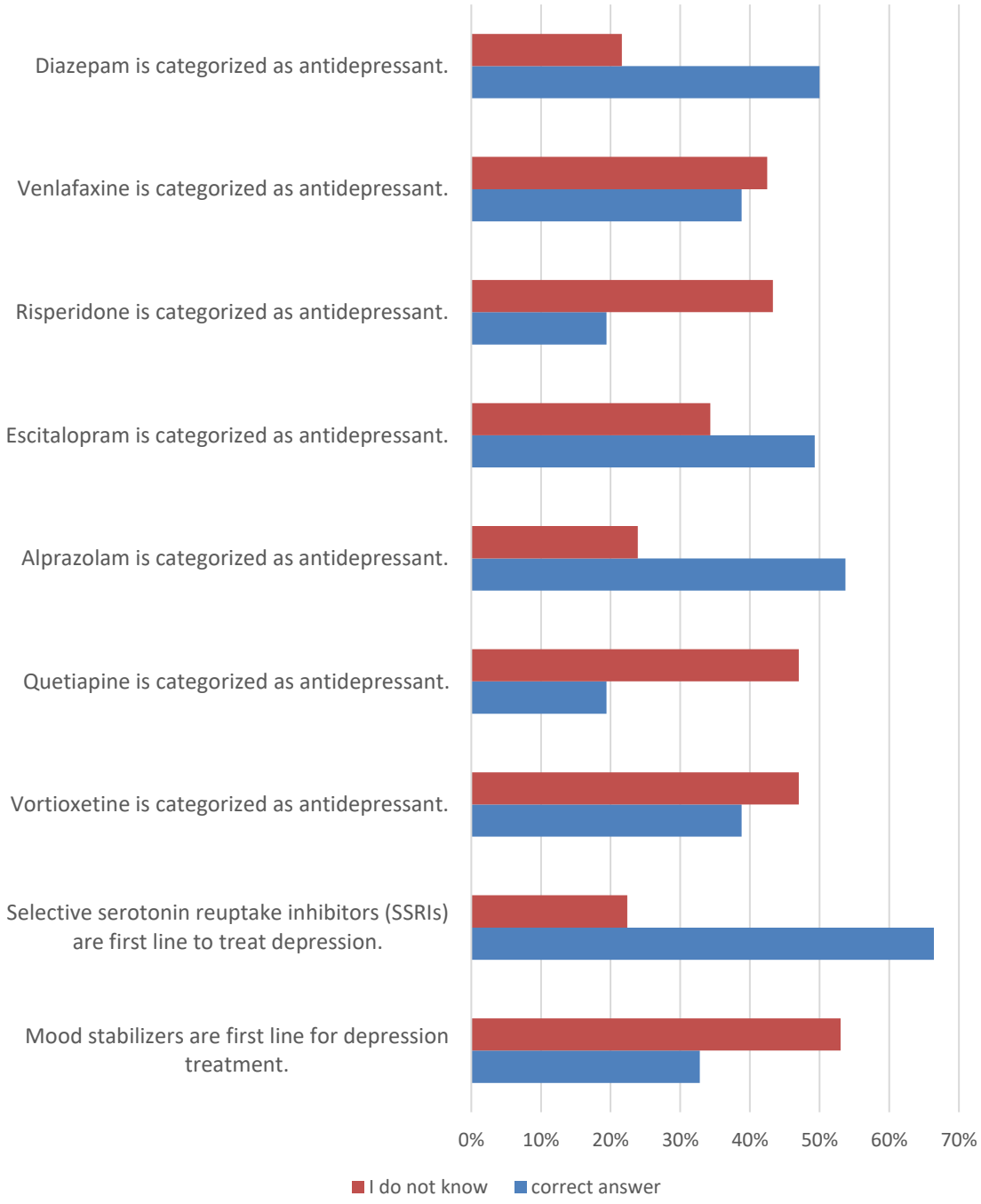


Fig 3 The knowledge of pharmacy students on psychotropic medications (n=134)



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تقييم تصورات طلاب الصيدلة للاكتئاب والأدوية النفسية في المملكة الأردنية الهاشمية

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ملخص

الخلفية: على الرغم من أن تعزيز معرفة طلاب الصيدلة وتصوراتهم للأمراض العقلية أمر مهم للتطوير الوظيفي في المستقبل، إلا أن مناهج الصيدلة في الأردن تعاني من نقص كبير في دورات الصحة العقلية ولم تدرس أي دراسة مواقف طلاب الصيدلة تجاه الأمراض العقلية ومعرفتهم بالأدوية العقلية في الأردن.

الهدف: تقييم معرفة طلاب الصيدلة بالاكتئاب والأدوية النفسية وتقييم مواقفهم تجاه توفير الرعاية الصيدلانية للمرضى الذين يعانون من الاكتئاب.

الطريقة: تم اجراء مسح عبر البريد الإلكتروني على عينة مؤلفة من 200 طالب صيدلية كانوا في السنة الرابعة والخامسة في جامعة أردنية واحدة. وتضمن الاستبيان أربعة أقسام؛ خصائص العينة، والمعرفة بشأن الاكتئاب، والموقف تجاه تقديم الرعاية الصيدلانية للمرضى الذين يعانون من الاكتئاب، ومعرفة طلاب الصيدلة حول الأدوية العقلية. تم استخدام اختبار Chi-square لتحديد العلاقات الهامة بين الترخصائص العينة والبيانات الأخرى.

النتائج: ورد ما مجموعه 134 ردا (معدل الاستجابة 67 في المائة). يعتقد معظم الطلاب أن المرضى الذين يعانون من الاكتئاب لن يتناولوا مضادات الاكتئاب إلى الأبد (ن = 104 ، 77.6٪). أقل من نصف (ن = 60، 44.8٪) من الطلاب يعتقدون أن مضادات الاكتئاب لا تسبب الإدمان. فقط حوالي ثلث (ن = 52، 38.8٪) من الطلاب يعرفون فينلافاكسين، و 47.0٪ من الطلاب (ن = 63) لا يعرفون فورتيوكسيتين. وارتبطت الدورات التدريبية في الطب النفسي ارتباطاً كبيراً بمعرفة طلاب الصيدلة في الطب النفسي (ص<0.05).

الخلاصة: على الرغم من أن الطلاب أعربوا عن موقف إيجابي تجاه PPC للمرضى الذين يعانون من الاكتئاب، يجب على طلاب الصيدلة تحسين معرفتهم بالعلاج الدوائي النفسي. وينبغي أن يدرج واضعو السياسات دورات عن الاضطرابات النفسية والعلاج الدوائي في المناهج الجامعية.

الكلمات الدالة: المواقف، الاكتئاب، الأمراض العقلية، طلاب الصيدلة، الرعاية الصيدلانية، الإدراك .

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Development of an Easy and Selective Approach for Determination of Titanium Dioxide in Commerical Cosmetics by UV-Vis Spectrophotometric Technique

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ABSTRACT

A simple and selective method was described for the determination of amount of titanium dioxide (TiO2) in cosmetics. The method is based on the complex formation of Ti(IV) with hydrogen peroxide in acidic media and monitoring the absorbance of the colored product by spectrophotometric technique at λ_{max}= 407 nm. The effect of experimental parameters on the reaction was investigated and optimized. Under optimal conditions, the calibration graph was linear in the range of 5.0-100 µg mL-1 of Ti (IV) with the limit of detection of 3.0 µg mL-1. The validity of the method was evaluated by means of the data statistical analysis. For this purpose, the method was applied to the determination of titanium dioxide in cosmetics, and the results were statistically compared based on t- and F-tests with those obtained by the Inductively coupled plasma-atomic emission spectroscopy (ICP-AES). There was no significant difference between the mean values and the precisions of the two methods at the 95% confidence level. The results showed that the proposed method offers an accuracy and reliable approach for the determination of TiO2 in commercial cosmetics, and can be suggested as a routine method in quality control laboratories.

Keywords: Titanium dioxide, Spectrophotometric, Cosmetics, Validation.

INTRODUCTION

Today, cosmetics have become an inseparable part of many people's lives, as cosmetics are substances that contain a wide variety of products that are used in face and body care, to accentuate or change a person's appearance. Thesis products including creams, powders, lotions, fragrances, lipsticks, lacquers, lenses, hair dyes, eye and face cosmetics, different types of oils and so forth.[1] The cosmetics industry has also grown to such an extent that anyone can access their desired products at any time. Due to the increasing growth of this field, and the importance of materials in the structure of cosmetics, quality control and their determination in cosmetic samples is essential.

Generally, cosmetics and personal care products contain a very sophisticated matrix with various components including minerals, herbal powders, oils, waxes, fats, colors, and ultraviolet (UV) preservatives.[2] There are many sunscreen cosmetics that use inorganic or organic UV filters as active ingredients.[3] Typical inorganic compounds in cosmetic or personal care are talc and metal oxides such as titanium dioxide, zinc oxide and iron oxide. Some organic compounds in cosmetics or personal care, can be naturally derived from modified fats and oils, or synthetically produced like petrochemical derivatives. Also, there are many cosmetics that, in addition to the beauty and protective effect of the skin, incorporating the organic or inorganic UV filters as active ingredients.[4] Chemically stable inorganic sunscreen agents, usually metal oxides, are widely employed in high sun protection factor (SPF)

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products.[5] One of the most important ingredients of cosmetics is titanium dioxide.[6] Titanium dioxide is a known photocatalyst and is used in the production of white pigments in the cosmetics and food industries.[7] In addition, It has been used as a pigment for a long period of cosmetic history.[8]

Inorganic compounds in cosmetics may undergo retention and act directly in the skin or be absorbed through the skin into the blood,[9] accumulate in the body and exert toxic effects in various organs.[10] Some cases of topical allergic contact dermatitis and systemic effects due to the metals present in cosmetics have been reported.[11]Also, studies of scientific data show that commercially available cosmetics products may contain large amounts of toxic metals,[12] which can be hazardous for human health.[13] For this reason, it is important to measure the ingredients in cosmetics in small amounts with a quick and easy method in cosmetic samples.[14]

The determination of the content of TiO_2 in sunscreen samples has been attempted with different techniques,[15] such as volumetry,[16] energy-dispersive X-ray fluorescence (EDXRF),[17, 18] transmission electron microscopy (TEM),[19] portable raman spectrometry,[20] atomic absorption spectroscopy (AAS),[21] X-ray fluorescence spectrometry., inductively coupled plasma-mass spectrometry (ICP-MS),[22] and inductively coupled plasma-atomic emission spectrometer (ICP-AES).[23] Although tool-based analytical methods are best for titanium analysis,[24] it is difficult for small cosmetics companies because of the high cost of analysis.[25] Spectrophotometric methods have a special place in the determination of titanium because of their simplicity, precision, and high sensitivity.[26-28]

Therefore, considering the important role of titanium dioxide in cosmetics, the aim of the proposed study is to evaluate the feasibility of using UV-Vis spectrophotometry as a simple, selective and user-friendly method for direct determination determine TiO_2 in cosmetics.

Experimental

Apparatus

UV-Vis spectrophotometer Model 3310 from Hitachi Company (Japan) with 1cm quartz cells was used for all the absorption measurements. All spectral measurements were performed using a blank solution as a reference. Magnetic stirrer (Hei Tec- Hidolph) and thermostatically controlled water bath (Model Hh-S4) were applied. Inductively coupled plasma -atomic emission spectrometer (Perkin Elmer PE Optima 5300DV) was used for comparison with a validation of the method.

Reagents and materials

All chemicals used in this study were analytical grade and used without any purification. Sulfuric acid, nitric acid, hydrogen peroxide (30% (w/w)), ammonium sulfate, and titanium dioxide were purchased with high purity from Merck. All cosmetic products were purchased from the cosmetics store in Tehran- Iran.

Experimental procedure

To prepare a stock solution of Ti(IV), an appropriate amount of TiO_2 was weighed and placed in a beaker. For dissolving and digestion of TiO_2, 4 g ammonium sulfate and 10 ml sulfuric acid were added. The mixture was heated to a boil until white vapors were observed. After that, the solution was cooled to room temperature and transferred to a 100 mL volumetric flask, and diluted to the mark with double distilled water. It was used as a stock solution (300 µg mL^{-1} Ti(IV)). A 150 mM of hydrogen peroxide solution was prepared from 30% (w/w) hydrogen peroxide solution. The inorganic solvent mixture was prepared by dissolving 4 g ammonium sulfate and 10 mL sulfuric acid in 100 mL deionized water and used for dilution of standard solutions.

For determination of TiO_2 quantities, under optimum conditions, aliquots of solutions containing Ti(IV), so that final concentration would be in the range of 5.0 –100 µg mL^{-1}, 1.5 mL of 150 mM of hydrogen peroxide solution were transferred into 10 mL volumetric flasks. After the formation of yellow color; the solutions were diluted to the

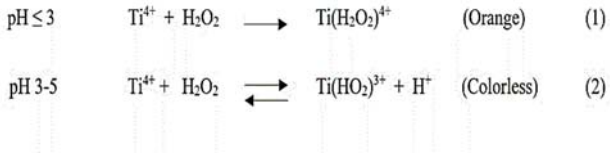
mark with the inorganic solvent mixture. A portion of the solution was transferred into a 1 cm quartz cell to measure the absorbance at 407 nm against a reagent as blank as the reference.

Sample preparation of commercial cosmetics for TiO_2 determination

In order to determine TiO_2 in cosmetics, the samples were placed in the furnace prior to analysis to burn the organic matter of the samples and give carbon-free ash. For this purpose, different weights of cosmetics were weighed on crucibles and placed in the furnace at 600 ° C for 2 h. After cooling of samples to room temperature, the acid digestion method was used to determine TiO_2. The method involved adding a mixture of 10 ml nitric acid (65% w/w) and 5 ml concentrated sulfuric acid to carbon-free ash cosmetic samples in a beaker and placing it in a water bath until the sample was dissolved. In order to complete the dissolution of the resulting solution, the beaker was heated to 200 ^{0}C until the dense fume of nitric acid was disappeared. Finally, 5 mL concentrated sulfuric acid and 4 gr ammonium sulfate were added to dissolve the TiO_2. The process was finished with the appearance of the white vapors. The resulting solution was cooled and carefully transferred into a 100 mL volumetric flask. The solution was diluted to the mark with double-distilled water.

Results and discussion

The reaction of Ti(IV) with hydrogen peroxide in an acidic media (pH≤ 3) gives a colored complex according to scheme 1.[29] Thus, it can be a successful method for the spectrophotometric determination of Ti.[30] Fig. 1 shows the absorption spectra of the complex which display a maximum absorbance at λ_{max}= 407 nm. Therefore, all examinations were performed at this wavelength. In order to achieve the highest performance and sensitivity, the influence of effective parameters on the reaction was investigated and optimum conditions were obtained. The optimization procedures were done by keeping all parameters to be constant and optimizing one each time. This optimization procedure may not lead to the actual optimum, but will definitely improve the analytical method. These parameters included the effects of concentration of reagent, the concentration of sulfuric acid, time, and temperature on reaction.



Scheme 1. Colored complex formation of Ti(IV) with hydrogen peroxide in an acidic media

Effect of the hydrogen peroxide concentration

The effect of hydrogen peroxide concentration on the absorbance of the system and determination of Ti(IV) was considered in the range of 50-200 mM. As Fig. 2 shows, the absorbance of the system was increased by increasing the concentration of hydrogen peroxide up to 150 mM and remain nearly constant at higher concentrations that show that the concentration of hydrogen peroxide has a tremendous effect on the formation of the color product between titanium and hydrogen peroxide. So, the concentration of 150 mM hydrogen peroxide reagent was selected as the optimum concentration for further investigation in the developed method.

Effect of the sulfuric acid concentration

The reaction between Ti(IV) and hydrogen peroxide takes place in acidic media. The importance and the influence of sulfuric acid concentration of absorbance of the product were studied in the range of 0.1-3 M of sulfuric acid. The results revealed (Fig. 3) that the absorbance increases by increasing the acid concentration up to 1.8 M and does not change at higher concentrations. Therefore, a concentration of 1.8 M sulfuric acid was applied in the proposed method.

Effect of temperature on the sensitivity

The effect of temperature on the formation of color product between Ti(IV) and hydrogen peroxide was investigated in the range of 15-60 °C was investigated. The

results showed that temperature has no effect on the reaction efficiency and this is one of the special advantages of the method, which makes it a temperature-independent and simple method which can be done without the need for heating equipment.

Study on stability of Ti(IV)-H_2O_2 complex

The influence of time on the complex formation of Ti(IV) with hydrogen peroxide was studied in a range of 1-10-min. The results showed that the reaction occurs immediately after the addition of hydrogen peroxide, and reaction of Ti(IV) with hydrogen peroxide is rapid and the reaction time is not critical. Also, it indicates that by following the absorbance at different times, the absorbance values were constant which shows that the Ti(IV)-H_2O_2 complex is a stable complex (Fig. 4). Therefore, the method has good repeatability and there is an improvement in figures of merit.

Effect of ionic strength

In order to study ionic strength on the reaction stability and resistance of the method, different concentrations of NaCl, KCl, Na_2SO_4, and KNO_3 solutions were separately added into the solution in the range of 150- 750 mM. The results show that the increasing the electrolytes concentration did not any effects on the absorbance of the system and stability of the Ti(IV)-H_2O_2 complex. Therefore, the proposed method to be robust, since no statistically significant differences were found when samples were subjected to these solutions.

Analytical performance

The analytical features that show the performance of the proposed method are presented in Table 1. The calibration graph was constructed by using different concentrations of standard solutions of Ti(IV) that subjected to the developed method and determined by the UV-Vis spectrophotometric method. Under optimum conditions, a calibration curve was linear in the range of 5-100 µg mL^{-1} of Ti(IV). The regression equation for proposed the method is, A= 0.0151 C +0.0066, with a regression coefficient (r) of 0.9994 (n=10), which *A* is the absorbance at λ max= 407 nm. The detection limit of the method, based on signal to noise ratio of 3, was 3 µg mL^{-1}.

Selectivity of method

To study the selectivity of the proposed method, the effect of various species on the determination of Ti(IV) was tested under the optimum conditions. For this purpose, sample solutions containing 45 µg mL^{-1} Ti(IV) and different concentrations of various ions were prepared and the developed procedure was applied. The tolerance limit was defined as the concentration of added species that caused a relative error of less than ±5%. The results showed that Na^+, K^+, NH_4^+, Ca^{2+}, Pb^{2+}, Co^{2+}, Sn^{4+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Fe^{2+}, Ca^{2+}, CO_3^{2-}, PO_4^{3-}, SO_4^{2-}, NO_3^-, Cl^-, Br^-, F^- did not interfere on the determination of Ti(IV) in a tolerance ratio (w s_{pecies}/w $_{Ti}^{4+}$) 500:1. This shows the good selectivity of the proposed method.

Statistical data analysis

The applicability of the developed method for the determination of Ti(IV) in cosmetic samples was investigated. Sample processing was discussed in the section of sample preparation. So, all cosmetics samples were subjected to the suggested method. The results of this investigation were shown in Table 2. The results revealed that the suggested method can be successfully applied for the determination of Ti(IV) in cosmetic samples. Validation and statistical comparison of the method were done with the determination of Ti(IV) in cosmetic samples by ICP-AES according to condition listed in Table 3.Also, the values of F-test and t-test were calculated, the values obtained from this study are less than the values stated in the Table. So, these results show that no significant differences between the results of the spectrophotometric method and the ICP-AES method at a 95% confidence level, and confirming the good accuracy and precision of the developed method.

Application of method in analysis of cosmetic samples

The reliability and matrix effects in the proposed method were investigated by the spiking-recovery method.

So different portions of cosmetic samples that prepared in the section of sample preparation were subjected with the spiking-recovery method and Ti(IV) was determined in both spiked and unspiked portions, and the relative recoveries (*R*) were calculated according to Eq. 3. :

$$Recovery\ (\%) = (C\_1 - C\_2)/C\_3 \qquad (3)$$

which C_1 the concentration of Ti(IV) after spiking of standard solution and C_2 is a real concentration of Ti(IV) in the sample solution, and C_3 is the concentration of Ti(IV) that spiked to sample solution. According to the results in Table 4 all recoveries in the range 94-103%. Acceptable recovery results can confirm that the matrix effect cannot have significant effects on the efficiency of the proposed method, and the results indicate that the proposed method has a high potential for the determination of TiO_2 commercial cosmetic samples.

Conclusions

Due to the fact that a lot of amounts of titanium dioxide can cause threaten human health. Therefore, monitoring the amount of titanium dioxide in cosmetic samples is important. Our investigation revealed that the proposed method is a simple, sensitive, and user-friendly spectrophotometric procedure for the determination of TiO_2 in a wide dynamic range that can be applied to cosmetics with satisfactory recoveries. Method validation shows that the method has high accuracy and precision and is robust. Therefore, this report can be proposed as a reliable method with high accuracy for the determination of titanium dioxide in cosmetics. Also, the method proved to be suitable for routine quality control of commercial cosmetics and can be extended for further study in this field.

Table 1. Optical and statistical parameters of regression equation and validation parameters

Molar absorptivity, Ɛ(L.mol^{-1} .cm^{-1})	**7.22 ×10^2**
Sandal's sensitivity (µg cm^{-2})	66.3
Color of complex	Yellow
Measurement wavelength [2]	407
Regression equation	A= 0.0151 C +0.0066
Regression coefficient (r^2)	0.9994
Slop of calibration curve	0.0151
Linear range (µg/mL)	5.0 – 100
Limit of detection(LOD= 3sb/m)	3.0 µg/mL

Table 2. Determination of TiO_2 in Cosmetic formulations by proposed method and comparison of it with ICP-AES method.

Cosmetics samples	Origin	Wieght of sample	Proposed method		ICP-AES method		t-values	F-value
			%Found in sample ± SD	RSD%	Found in sample ± SD	RSD%	(2.78)[a]	(9.28)[a]
Paint stick cream	**USA**	0.2	9.03 ± 0.015	0.16	9.05 ± 0.04	0.44	1.33	7.11
		0.3	9.05 ± 0.073	0.80	9.07 ± 0.036	0.40	0.57	4.11
		0.4	9.07 ± 0.108	1.19	9.08 ± 0.045	0.50	0.2	5.76
BB-cream	**France**	0.2	4.51 ± 0.041	0.90	4.54 ± 0.036	0.79	1.5	1.29

Cosmetics samples	Origin	Wieght of sample	Proposed method		ICP-AES method		t-values	F-value
			%Found in sample ± SD	RSD%	Found in sample ± SD	RSD%	(2.78)[a]	(9.28)[a]
		0.3	4.49 ± 0.036	0.80	4.58 ± 0.017	0.37	3.60	4.48
		0.4	4.51 ± 0.047	1.03	4.53 ± 0.041	0.90	0.76	1.31
Cake make-up	**France**	0.2	2.15 ± 0.033	1.54	2.13 ± 0.041	1.92	1.0	1.54
		0.3	2.16 ± 0.026	1.20	2.14 ± 0.031	1.44	1.17	1.42
		0.4	2.15 ± 0.026	1.23	2.14 ± 0.025	1.16	0.66	1.08
		0.2	3.49 ± 0.026	0.74	3.48 ± 0.10	0.28	0.21	7.01
Foundation cream	**France**	0.3	3.46 ± 0.036	1.04	3.47 ± 0.017	0.143	0.66	6.0
		0.4	3.50 ± 0.017	0.49	3.51 ± 0.01	0.28	0.83	2.0
		0.2	5.69 ± 0.030	0.73	5.67 ± 0.10	0.25	1.21	6.01
Toothpaste	**Iran**	0.3	5.56 ± 0.039	1.17	5.58 ± 0.017	0.151	0.87	7.0
		0.4	5.30 ± 0.018	0.53	5.32 ± 0.01	0.24	0.76	2.1

[a] It is the theoretical value based on the paired *t*-test and F-test at the level of significance of $p = 0.05$

Table 3. Operating conditions of ICP-AES

Operating conditions of ICP-AES	
Parameter	**Value**
Reflected power	1350 W
Radiofrequency (RF) generator	40 MHz, free-running
Auxiliary gas flow rate	0.50 L min^{-1}
Viewing mode	Axial
Torch type	Fassel type
Injector, id	Alumina, 2.0 mm
Nebulizer	Gem tip cross flow
Sample uptake flow rate	2 mL min^{-1}
Delay time	30 s
Wavelength	334.94 nm
Plasma gas flow rate	15 L min^{-1}

Table 4. Real samples analysis results

Cosmetics samples	Ti^{4+} (µg mL^{-1})		
	spiked	Found (µg mL^{-1}) ± [a]SD	Recovery%
TV paint stick	0.0	27.15 ± 0.015	-
	15	42.66 ± 1.52	103.4
	30	57.06 ± 0.89	99.7
	35	63.00 ± 1.00	102.4

Cosmetics samples	Ti^{4+} (µg mL^{-1})		
	spiked	Found (µg mL^{-1}) ± [a]SD	Recovery%
BB-cream	0.0	18.21 ± 0.026	-
	15	32.33 ± 1.52	94.13
	30	47.8 ± 0.75	98.63
	45	63.53 ± 0.64	100.7
Cake make-up	0.0	12.79 ± 0.014	-
	15	27.55 ± 0.32	100
	30	43.03± 0.139	100.7
	45	58.3± 0.36	101.1
Foundation cream	0.0	14.1 ± 0.1	-
	15	29.56 ± 0.25	103.73
	30	44.73 ± 0.27	102.43
	45	58.5 ± 0.25	98.88
Toothpaste	0.0	13.1 ± 0.11	-
	15	28.54 ± 0.25	104.72
	30	43.73 ± 0.26	100.54
	45	62.00 ± 1.00	99.67

[a] Average of three determinations± standard deviation.

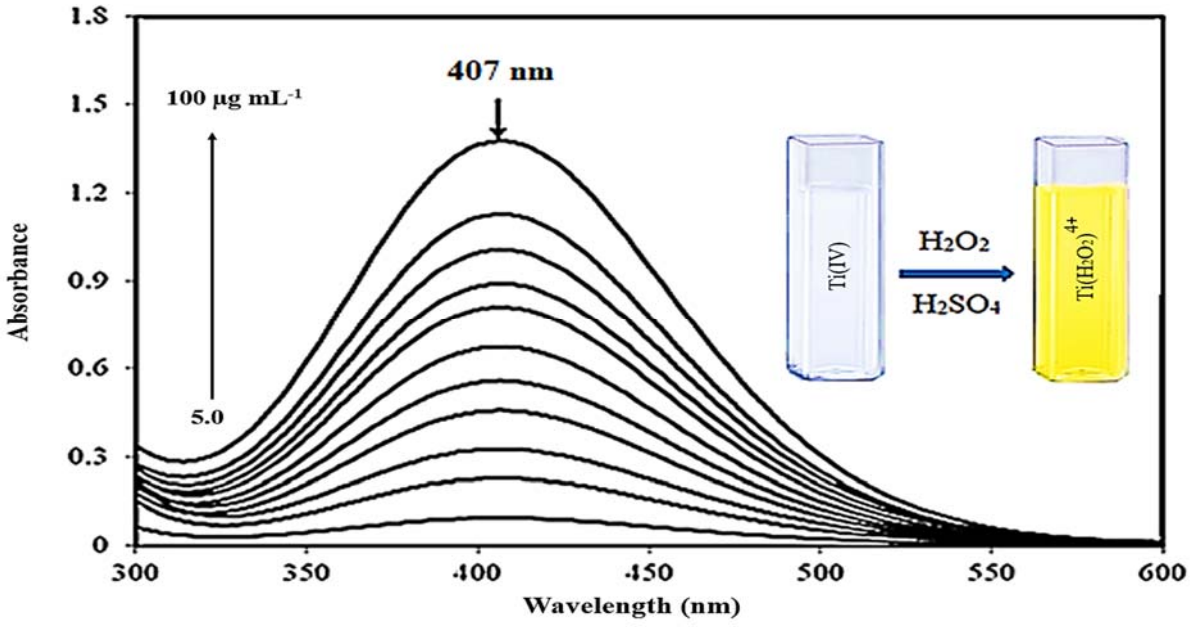


Figure 1. Absorption spectrum resulting from titanium and hydrogen peroxide reaction, Conditions: Concentration of Ti(IV): 5-100 µg mL^{-1}, hydrogen peroxide: 150 mM, sulfuric acid: 1.8 M, temperature: 25 ^{0}C

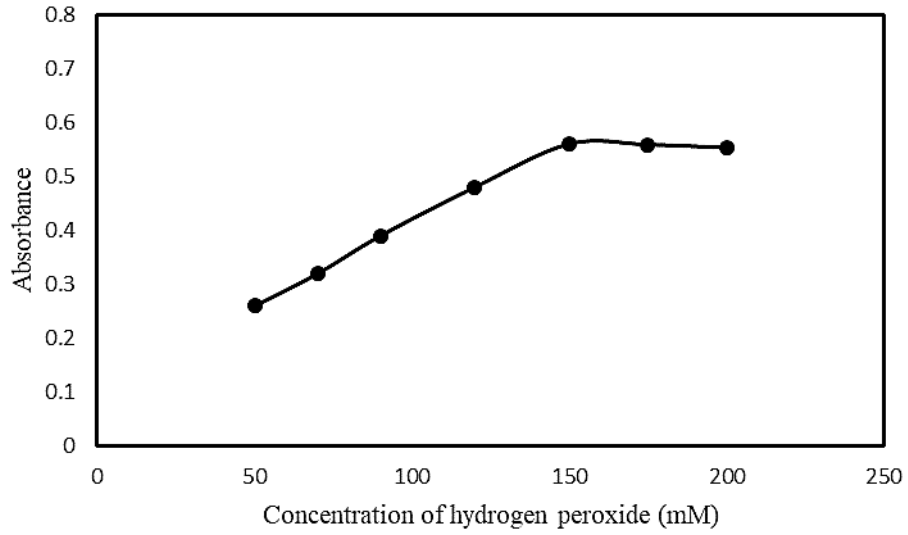


Figure 2: Effect of concentration of hydrogen peroxide on reaction, Conditions: Ti(IV): 45 µg mL^{-1}, sulfuric acid: 1.8 M, temperature: 25 ^{0}C.

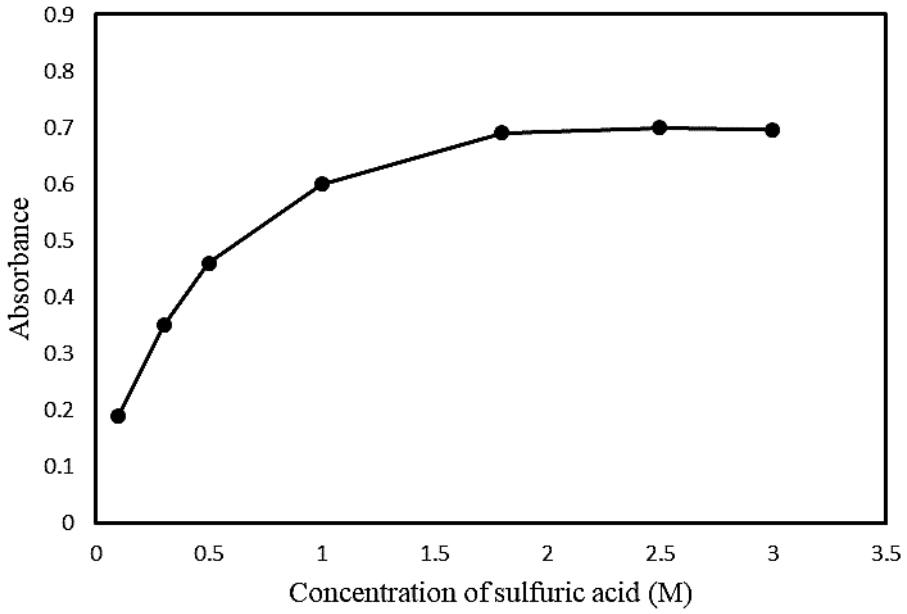


Figure 3: Effect of concentration of sulfuric acid on reaction, Conditions: Ti(IV): 45 µg mL^{-1}, hydrogen peroxide: 150 mM, temperature: 25 ^{0}C.

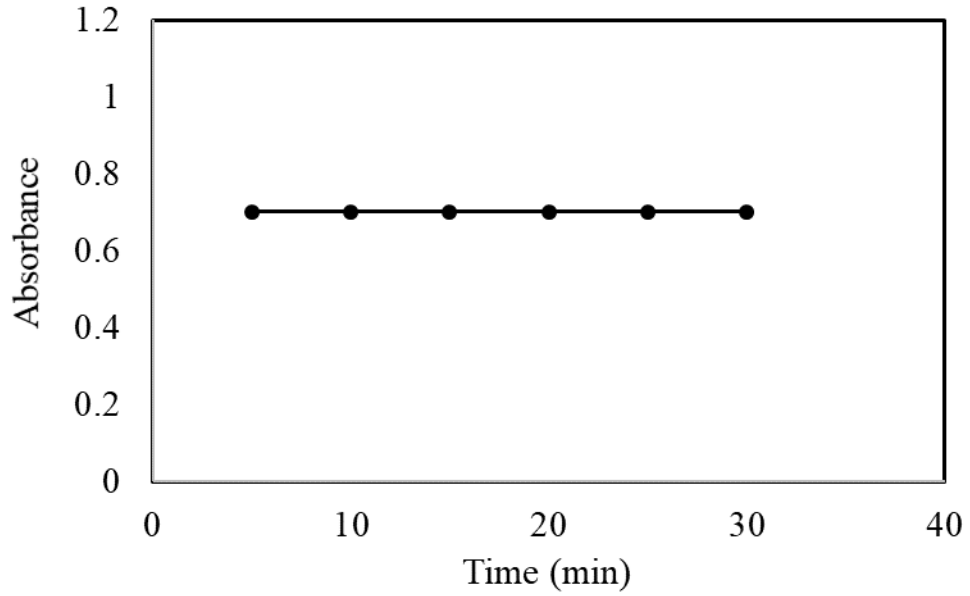


Figure 4: Effect of time on reaction, Conditions: Ti(IV): 45 µg mL^{-1}, hydrogen peroxide: 150 mM, sulfuric acid: 1.8 M

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تطوير نهج سهل وانتقائي لتقدير ثاني أكسيد التيتانيوم في مستحضرات التجميل التجارية باستخدام تقنية قياس الطيف الضوئي بالأشعة المرئية وفوق البنفسجية

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ملخص

تم وصف طريقة بسيطة وانتقائية لتقدير كمية ثاني أكسيد التيتانيوم (TiO2) في مستحضرات التجميل. تعتمد الطريقة على التكوين المعقد لـ Ti (IV) مع بيروكسيد الهيدروجين في الوسط الحمضي ومراقبة امتصاص المنتج الملون بتقنية القياس الطيفي عند λmax = 407 نانومتر. تم دراسة تأثير المتغيرات التجريبية على التفاعل وتحسينه. في ظل الظروف المثلى، في ، كان الرسم البياني للمعايرة خطيًا في نطاق 5.0–100 ميكروغرام مل من Ti (IV) مع حد اكتشاف 3.0 ميكروغرام مل. تم تقييم صحة الطريقة عن طريق التحليل الإحصائي للبيانات. لهذا الغرض ، تم تطبيق الطريقة لتقدير ثاني أكسيد التيتانيوم في مستحضرات التجميل ، وتمت مقارنة النتائج إحصائيًا بناءً على اختبارات t− و F مع تلك التي تم الحصول عليها عن طريق التحليل الطيفي للانبعاثات الذرية بالبلازما المقترنة حثيًا (ICP-AES). لم يكن هناك فرق كبير بين القيم المتوسطة ودقة الطريقتين عند مستوى ثقة 95٪. أظهرت النتائج أن الطريقة المقترحة تقدم طريقة دقيقة وموثوقة لتحديد TiO2 في مستحضرات التجميل التجارية ، ويمكن اقتراحها كطريقة روتينية في مختبرات مراقبة الجودة.

الكلمات الدالة: ثاني أكسيد التيتانيوم، مقياس الطيف الضوئي، مستحضرات التجميل، التحقق من الصحة.

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The Relationship between Family Socialization Patterns and Attitudes towards Drug Use among Users and Addicts of Cannabis and Alcohol Who Wish to be Treated in Private Clinics in Amman, Jordan

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ABSTRACT

This study aims to examine family socialisation patterns with attitudes towards drug use among addicts to Cannabis and alcohol, seeking treatment in private clinics in Amman, Jordan. The study employed the quantitative research design, and the sample of the study consists of 45 male participants, where 19 were alcohol users and 26 were hashish users. The participants were selected based on convenient sampling from different private clinics. Every individual in the two groups was subjected individually. To achieve the study's objective, the researcher adopted two scales: attitudes towards drugs and narcotics abuse scale, and family socialisation scale. The study findings showed that the highest mean of addiction was for the pattern of democratic, and the correlation coefficients between addiction and family socialisation are statistically significant. Besides, the relationship is negatively strong between addiction and the democratic pattern, while the relationship is positive between addiction and both the authoritarian and lenient socialisation pattern. Also, results showed that the patterns of family socialisation explained 72% of the prediction of addiction, while the square of the correlation coefficient (R^2) is (0.520), with an explanatory ability of (49%) in predicting addiction.

Keywords: Family Socialization patterns; drug use among users; addicts; Cannabis; alcohol.

INTRODUCTION

The American Psychological Association in DSM-5 defined alcohol use disorder as alcohol abuse that a person does not control, leading to significant impairment or distress. Moreover, it often refers to the consumption of alcohol in greater amounts or over a longer period with permanent desire or unsuccessful efforts to stop or control alcohol use [1]. The term also refers to those who spend a great deal of time on activities necessary to obtain or use alcohol. This type of addiction impedes the person from adhering to his daily life, working, studying responsibilities, or performing social partnerships, especially that the person might continue to use it despite health, social or personal problems due to persistence of alcohol use [2, 3].

Alcohol is used when engaging in activities that are physically dangerous [4]. The World Health Organization defines alcoholics as individuals who drink excessively to the extent that leads to the emergence of a noticeable mental disorder, disrupting their physical and psychological health, their relationship with others, and their social and economic functions. This is because alcohol impedes the nerve cell activity, especially the cerebral cortex, and it quickly penetrates the central nervous system because it quickly dissolves in water and begins to act within an hour of starting use [5, 6].

Besides, drugs are defined as every natural or industrial substance that contains stimulant or analgesic substances if used for purposes other than medical purposes that lead to a state of psychological or physical

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dependence/addiction [7, 8]. It is harmful to the psychological or physical health of the individual and society, and it is prohibited from circulating or cultivation or manufacture, except for purposes determined by the law and used only by someone authorised to do so [9, 10].

In terms of family socialisation, it means the procedures and methods used by parents in socialising their children, i.e. transforming them from mere biological beings into social beings by directing their behaviours and providing them with knowledge, patterns of behaviour, values, symbols, and ways of dealing and thinking [11, 12]. Family raising might be affected by external or internal factors. The external factors are related to the general cultural framework of society, as the individual lives in a cultural framework consisting of the customs, traditions, and beliefs in which the individual is formed and socialised with different values and customs. Also, internal factors, including the relationship, marital and social position, and family size, affect the parenting trends followed by parents in the upbringing of their children [13].

The importance of the study lies in its treatment of a dangerous phenomenon that affects the whole substance abusers. According to [14], it is of great necessity to study family upbringing since it has a primary role in the direction of the abuser, and the family might play a negative role in leading their children to drug addiction. That is, the psychological conflict, immaturity and inability to enjoy life is the result of the authoritarian style, and it aids children to escape from the restrictions and domination from this conflict to the drug addiction [15]. Also, punishment, deprivation, and constant threats create an atmosphere of non-intimacy among family members, so children resort to companions outside the home to practice their activities more freely, which increases the chances of addiction among children [16]. Besides, the pattern of excessive care in upbringing may lead to the inability to take responsibility and emotional instability among children, making children lacking self-confidence and suffer from constant fear; therefore, children escape from such psychological pressures to addiction behaviours [17].

Accordingly, the current study investigates the relationship between family socialisation patterns and attitudes towards drug use among users and addicts of Cannabis and alcohol, who seek treatment in private clinics in Amman, Jordan. This aim is investigated through the following questions:

1. Is there a statistically significant correlation between the patterns of family socialisation and trends towards drug use among the participants?
2. Are there statistically significant differences in means of the patterns of family socialisation among users and addicts of Cannabis and alcohol?
3. What is the percentage of variation that can be explained by the pattern of family socialisation in predicting the use and addiction of Cannabis and alcohol among the participants?

LITERATURE REVIEW

Many studies have dealt with family socialisation and have concluded that the specific use of Cannabis increases health and family problems. Alcohol use, which was not reflected in general drug use, had no specific negative effects, but it reduced loneliness in romantic relationships, self-derogation, and family problems. The vital role of self-perception, whether this derives from a sense of self through practice or participation in social groups, might increase drug use [18]. Drug use may resemble a learning curve where drug-using peers are ascribed to attract drug users. The perception of addiction as problematic is mainly related to heroin infringing upon all life domains. Entering treatment was found to be sometimes unrelated to the decision to quit drug use. Another study reported that the form of family socialisation to which the individual is exposed has an essential role in predicting the normal or abnormal behavioural patterns that the individual will exercise in the future [19].

Moreover, the awareness of the family and its role as a watchdog for children contribute very significantly to

reducing drug use to enhance social security[20]. The family interest in socialising and educating their children about the harmful effects of drugs contributes considerably to reducing their abuse and strengthening social security. For example, girls who suffer from discrimination within the home and face insulting, beating, and neglect, can resort to drug addiction [21, 22].

Past studies have discussed two-family patterns, namely the democratic pattern, which is one of the normal patterns, and the authoritarian pattern, which appears through punishment, deprivation, and threat, and their treatment is based on the principle of violence with the symbolic, physical and psychological problem[23]. Also, oppression is followed as a way of life in general, while the third pattern is the pattern of excess protection, and this pattern is manifested in parents' concern to take duties on behalf of their children, who become unable to face reality and the pressures of the environment. Such attitude also negatively affects the child's emotional stability and self-confidence, so they have constant fear due to the inability to take responsibility and eventually become introverted and isolated [24]. The pattern of negligence leaves the children without care or encouragement for the desired behavior, so they cannot avoid their unwanted behaviour because they lack the sense of life responsibility, which alters them unintentionally to the wrong behaviour [25].

Further, socialisation takes two main forms, including intended socialisation, which takes place in both the family and the school. According to its cultural system, standards, and directions, the family teaches the children language, etiquette, and behaviour [26]. Also, school education in its various stages must be intended by having goals, methods, systems, and curricula that relate to the education of the individual and its development in a specific and intended way [27]. However, the other type is unintended socialisation, which is carried out to accompany the intended upbringing. This pattern is often formed in through mosque, media, radio, television, cinema, theatre, and other institutions that contribute to the upbringing by the rules through which the individual learns the skills, meanings, and ideas, especially that social norms differ from one institution to another [28].

There are also personal factors that relate to the parents themselves, such as their personality style, educational level, and the way parents are treated and brought up in their childhood [29]. Among the family patterns is the democratic style that is the opposite of the authoritative style. The democratic family is characterised by excessive protection and negligence for the children's behaviour. Also, parents might not be direct in their treatment with their children or tend to be injustice with them, which leads to addiction [30].

To conclude, the family greatly influences drug addiction, and many family factors lead young to drug addiction [21]. Therefore, the current study investigates the role of family socialisation patterns in drug addiction since family socialisation patterns greatly influence individual behaviours and practices.

METHODS AND MATERIALS

a. Participants

The sample of the study consists of45 male participants, who were selected based on convenient sampling from different private clinics, (19 alcohol users and 26 hashish users). The participants' age ranged between 18 and 45 years. Every participant in the two groups was individually subjected to the surveys.

The sample was selected from males due to the lack of female visitors to the clinics where the test was applied. Also, the study used convenient sampling technique to select the participants since the selection depended on the sample available to the researcher in the three clinics. Some participants refused to participate in the study due to their fear that this research is affiliated with security authorities even though the researcher has clarified the nature of the research and the professional ethics of psychologists, and he clarified that they do not need to write their names on the paper of surveys' responses. Also,

there was a difficulty in finding alcohol addicts who have not used hashish or any other substance.

The exclusion criteria were for those who abused different substances in addition to Cannabis or alcohol, and those who refused to participate in the research. The number of hashish abusers who were excluded was 23 persons, and 7 persons are alcohol addicts, but they refused to submit their responses to the study. The reason for their refusal was their lack of confidence in the researchers, and that this method might be used by some security authorities to reach the addicts.

One of the difficulties facing the researcher is the method of obtaining the sample, so the number of the sample participating in the research was small, especially that exclusion was made for those who use or are addicted to substances other than alcohol and hashish, or who mix in using more than one drug. This is to ensure the sample homogeneity in terms of abuse and addiction.

b. Study Instruments

After reviewing previous studies and different scales that searched in variables of the current study, the researcher adopted scales according to the following:

1. Attitudes towards Drugs and narcotics abuse. The scale consists of 38 items that represent the emotional side, which are feelings of pleasure, joy, anger, hate and annoyance that lead to the individual accepting the use of dangerous drugs and drugs.

2. The family socialisation scale was created by Buri[31], and it includes 30 items divided into three dimensions of family socialisation: the lenient style, the authoritarian style, and the democratic style.

The language of the current study's instruments was Arabic. The scale of attitudes towards alcohol and drugs is prepared by the researcher Ahmed Mahmoud Abu Ein (Abu Ein 2008) in Arabic language. However, the researcher in the current study has tested the validity of the two scales as discussed in the section below.

Four items of the scale of attitude towards alcohol and drugs have been adapted. The changes were made to items number 4, 12, 14, and 31. In item number 4, the word "companions" has been replaced by the word "friends". In item number 12, the phrase "my comanions" is replaced by the word "friends". In item 14, the phrase "dangerous substances" has been replaced by the phrase "alcohol and drugs". In item 31, the word "companions" has been replaced by the word "friends".

c. Validity and Reliability

The researcher relied on checking the validity and reliability of the scales on eight experts in the field of psychology to judge the suitability of each item of the scales. The judges agreed that the expressions used in all items are clear and appropriate for what they intend to measure, and hence the scales are valid for application.

The reliability of the two instruments has also been re-tested through piloting the two instruments to 15 participants. The reliability of Cronbach's Alpha using SPSS showed that the attitude scale value is 0.793 and the family socialisation scale value was 0.764, which shows that the internal consistency of the two scales is good.

d. Data Analysis

The current study will use Statistical Package of Social Sciences (SPSS) to analyse the data. To answer the first research question about the significant correlation between the patterns of family socialisation and trends towards drug use, the researcher used Pearson correlation coefficients between family socialisation patterns and addiction. For research question two, the researcher will use independent sample t-test to investigate if there statistically significant differences in means of the patterns of family socialisation among users and addicts of Cannabis and alcohol. Research question three investigates the percentage of explained variation that can be explained by the pattern of family socialisation in predicting the use and addiction of Cannabis and alcohol between the participants, which was analysed through regression analysis.

e. Research Procedures

Researcher procedures included the following:

1. The patients were selected from the following

clinics: Creative Minds Clinic, Al-Murad Mental Health Clinic, and Dr. Zuhair Al-Dabbagh Clinic. These clinics are specialised for the treatment of psychological illnesses and addiction.

2. Each clinic is visited by an average of 5-6 patients per day for various mental illnesses, including addiction.

3. The addict was interviewed in the clinic in which he is referred, and the standards were applied to the subject in the same clinics in which he is visited.

4. After the patient is diagnosed as addicted to alcohol or to Cannabis, the patient is requested to participate in the research, assuring him that the data is confidential and will used for research purposes only.

5. Each clinic visitor is assigned to an interview in the same clinic.

6. The patient is called later on any day or on the same day on which he met the doctor.

7. The study was implemented in 2019.

8. The researcher has informed the participant about the purpose of the study and the complete confidentiality in which data is treated. To increase the confidence of the addict, he was not asked to write a consent form due to the sensitivity of the topic of the study.

9. The participants did not write their names on the study instruments to maintain complete confidentiality, and the participant responded to both scales in a separated room.

10. Psychiatrists diagnose patients by relying on the criteria on which the DSM-5 book is based.

11. Privacy was taken into account in all procedures for applying the research, and the ethical aspects of scientific research were taken into account during data collection.

12. The researcher has made sure to clarify the purpose of the study for the participants, clarify complete confidentiality, and clarify any ambiguous items in the scales.

13. A minute was given for each paragraph to be answered by the examinee. The two scales have 68 items, so 68 minutes were given to the participants to respond to the items of the two scales.

RESULTS

In terms of the demographic information, the number of the participants of the current study were 45 persons, including 26 cannabis addicts and 19 alcohol addicts. Their age mean is 28.4 and 27.2 for cannabis addicts and alcohol addicts, respectively. Also, the range of age were between 17 to 42 for alcohol addicts, and between 21 to 38 for hashish addicts. The age of the participants is shown in Table (1) below.

Table 1. Demographic information of participants

	Alcohol Addict	**Hashish Addict**
No.	19	26
Age mean	28.4	27.2
Range of age	17-42	21-38

Before moving to answer every research question, the descriptive analysis of the attiude towards towards drugs and narcotics abuse for the three styles of family (the lenient style, the authoritarian style, and the democratic style) is analysed according to the responses of the respondents. The scale consists of 30 items distributed equally among the three family styles, and there are 5 degrees to answer each item. The sub-score for each type was 10 items, the authoritarian style 10 items, the lenient style 10 items, and the democratic style 10 items. The higher the grades of the family style, the more his parents are from this pattern of family. All items were positive, not negative. The mark for each item ranged from 10 to 50. The findings shows that there are no differences in family upbringing between abusers and addicts to hallucinogens and sedatives. However, the results shows that the abusers and addicts to hallucinogens had a greater tendency towards addiction compared to drug abusers and addicts to sedatives.

1. Is there a statistically significant correlation between the patterns of family socialisation and trends towards drug use among the participants?

The result of the analysis of this research question is

shown in Table (2), which shows that all the correlation coefficients between addiction and family socialisation are statistically significant. That is, the p-values of the relationship between addiction and the patterns of family socialization are .003, .001 and .000 for authoritative, democratif and lenient, respectively.

Table 2. Pearson correlation coefficients between family socialisation patterns and addiction

	Authoritative	Democrat	Lenient
Addiction	.428**	-.489**	.606**
N	45	45	45
Sig	.003	.001	.000

*Significant at ($\alpha \leq 0.01$)

2. Are there statistically significant differences in means of the patterns of family socialisation among users and addicts of Cannabis and alcohol?

The findings of this research question is shown in Table (3), which indicates statistically significant differences between abusers and addicts of Cannabis and alcoholdue to the pattern of family upbringing in the authoritative and democratic types of family socialization with p-values of .000 for both of them. However, there were no statistically significant differences between abusers and addicts attributed to the lenient family socialization since the p-value is .438, which is not significant as the level of .05.

Table 3. Results of t-test for independent samples were tested

Pattern	Addicts	N	SD	M	F	t	Sig
Auth	Can.	26	7.2943	24.39	.000	7.438	43
	Alc.	19	6.274	39.84			
Dem	Can.	26	6.741	40.00	.000	4.215	43
	Alc.	19	7.605	30.95			
Len.	Can.	26	3.939	14.65	.438	.782	43
	Alc.	19	3.892	15.58			

3. What is the percentage of explained variation that can be explained by the pattern of family socialisation in predicting the use and addiction of Cannabis and alcohol between the participants?

In addition to the statistics of means and standard deviation in Table (3) and results of Pearson correlation coefficients that showed the relationship between family socialisation patterns and addiction in Table (2), the results of the regression analysis to answer this research objective (3) is provided in Tables (4). That is, the Linear regression analysis was used to find the relationship between family socialisation patterns and addiction as shown in Tables (4).

Table 4.Results of linear regression analysis of patterns of family socialisation in the prediction of addiction.

Indep. variables	Regression coefficient B	Stand. Error	β	T	Sig.
Constant	157.013	31.915	-	4.920	.000
Auth.	-1.561	.364	-.506	-4.288	.000
Democrat	.641	.485	.169	1.322	.193
Lenient	-2.488	.960	-.306	-2.593	.013

According to Table (4), results of the correlation coefficients between addiction and family socialisation are statistically significant, and the relationship is positively strong between addiction and the democratic pattern. However, the relationship is negative between addiction and both the authoritarian and lenient socialisation pattern. Hence, these patterns can predict the level of addiction in a significant way.

Also, the patterns of family socialisation explain 72% of prediction of addiction since the correlation (R) value is .721, while the square of the correlation coefficient (R^2) is (0.520); with an explanatory ability (49%) in predicting addiction.

DISCUSSION

This study was designed to investigate the relationship of family socialisation patterns with attitudes towards drug use among users and addicts of Cannabis and alcohol who wish to be treated in private clinics in Amman Jordan. The study findings showed that the highest mean of addicts was for the democratic style with a mean value of (36), followed by the authoritarian style with a mean value of (31) and then the lenient style with a mean value of (15). The most common socialisation pattern among addicts was the democratic and authoritative pattern, and correlation coefficients between addiction and family socialisation are statistically significant. Also, the relationship is negatively strong between addiction and the democratic pattern, while the relationship is positive between addiction and both the authoritarian and lenient socialisation pattern. The results showed that the patterns of family socialisation could explain 72% of the prediction of addiction, while the square of the correlation coefficient (R^2) is (0.520), with an explanatory ability (49%) in predicting addiction.

These results can be explained by the role of the family in the socialisation process, since the family abandons its functions and loses its structural balance due to poor parental control over the children or because of the absence of one of the parents. Such families tend to use either an authoritarian or lenient socialisation pattern. Both of them lead to a weak socialisation process for the children and not to produce a normal generation that has a healthy psychological development [32].

The addiction increases with the dominance of family socialisation patterns and lenient; however, it is decreased with the democratic family socialisation pattern. This correlation results from the interaction of the family socialisation process with human behaviours. According to [33], the process of socialisation focuses on what a person learns. It is a process that helps build the human personality to gain experience as it is influenced by the culture of the society in which the person lives. Society includes the family, school, and other socialisation institutions as well as friends. The learning process consists of a wide range of behaviours, some of which are positive, such as cooperative behaviours and honesty, and there are negative ones such as addiction and violence [34]. In this context, child abuse by parents during childhood contributed to the prediction of misbehaviour later, and exposure to the experiences of abuse is one of the most unwanted results of the authoritative and lenient socialisation patterns [35].

Besides, family awareness is essential for children to improve social security, which is an important factor in reducing addiction [20]. Family education and socialisation make children feel attached to their family, so they do not involve themselves in any misconduct to compensate for the loss of being attached to their families. This is also related to the community's general cultural and social framework, which provides people with values and ethics [36]. Such factors also require socialisation and good bringing from parents, and these factors play a main role in preventing young from being involved in drug use [13]. An important point is that family socialisation with individuals helps to understand the needs and psychology of the family members, leading to predicting any false behaviour that might be exercised by any of the family members [19, 36]. Hence, family socialisation is needed to

protect the individual from being involved in drug use, and it is equally important to deal with such problems properly in the early stages of addiction.

The role of the family is needed to change any false behaviours or ideas that might make the individual follow the dark tracks of drug use [36]. So, treatment programs are necessary for those subjected to abuse to treat false ideas that cause misbehaviour [35]. These ideas need to be replaced with peaceful and healthy thoughts. In this context, the role of family socialisation is of great importance to asset individuals to go back to their normal life and overcome drug addiction.

Limitations and Directions for Future Studies

The findings of this study should be generalised with caution as it has some limitations like any other study. The context of the study was restricted to Jordanian participants, and it investigated a limited number of variables. Therefore, future studies are advised to employ a larger sample size and explore other independent variables that might mediate substance abuse. Also, future studies might employ a mixed-method design since qualitative data can support the quantitative findings to provide a deeper understanding concerning family socialisation and drug addiction.

Conclusion and Implications

This current study aimed to investigate the relationship between family Socialisation patterns and attitudes concerning drug use among two groups, namely users and addicts of Cannabis and alcohol, and are seeking treatment in private clinics in Amman, Jordan. The findings showed that the democratic style scored the highest mean. Further, the findings showed that the relationship is negatively strong between addiction and the democratic pattern. In contrast, the relationship is positive between addiction and both the authoritarian and lenient socialisation pattern. An important point is that the patterns of family socialisation explained 72% of the prediction of addiction.

The findings can give new insights on how to deal with substance abuse and can further offer implications to help us mitigate the negative effects of substance abuse. The results showed that family socialisation is a key aspect that can help prevent substance abuse, which put a crystal-clear implication for the role of the family in mitigating and even preventing substance use. Hence, addicts need more care and support from the side of their families to help them avoid being involved in drug addiction.

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العلاقة بين أنماط التنشئة الاجتماعية الأسرية والمواقف تجاه تعاطي المخدرات بين متعاطي ومدمني الحشيش والكحول الذين يرغبون في العلاج في العيادات الخاصة في عمان، الأردن

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ملخص

هدفت هذه الدراسة إلى فحص أنماط التنشئة الاجتماعية الأسرية مع المواقف تجاه تعاطي المخدرات بين مدمني الحشيش والكحول، الباحثين عن العلاج في العيادات الخاصة في عمان، الأردن. استخدمت الدراسة تصميم البحث الكمي، وتتكون عينة الدراسة من 45 مشاركًا من الذكور، حيث كان 19 من مستخدمي الكحول و 26 من مستخدمي الحشيش. تم اختيار المشاركين بناءً على عينات ملائمة من عيادات خاصة مختلفة. تعرض كل فرد في المجموعتين بشكل فردي. ولتحقيق هدف الدراسة اعتمدت الباحثة مقياسين: مقياس الاتجاهات نحو تعاطي المخدرات والمخدرات، ومقياس التنشئة الاجتماعية الأسرية. أظهرت نتائج الدراسة أن أعلى متوسط للإدمان كان للنمط الديمقراطي، وأن معاملات الارتباط بين الإدمان والتنشئة الاجتماعية الأسرية ذات دلالة إحصائية. إلى جانب ذلك، فإن العلاقة قوية بشكل سلبي بين الإدمان والنمط الديمقراطي، في حين أن العلاقة إيجابية بين الإدمان ونمط التنشئة الاجتماعية السلطوي والمتساهل. كما أظهرت النتائج أن أنماط التنشئة الاجتماعية الأسرية فسرت 72٪ من التنبؤ بالإدمان، بينما مربع معامل الارتباط (R^2) هو (0.520)، بقدرة تفسيرية (49٪) في التنبؤ بالإدمان.

الكلمات الدالة: انماط التنشئة الاجتماعية الاسرية، اساءة استخدام المخدرات، الادمان، الحشيش، الكحول.

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Activity of Isoxazole substituted 9-aminoacridines against SARS CoV-2 main protease for COVID19: A computational approach

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ABSTRACT

Coronavirus Disease 2019 (COVID-19), a life-threatening viral disease affected first in china and quickly spread throughout the world in early 2020. So many scientists are rushing to discover novel drugs and vaccines against the coronavirus, and treatments for COVID-19.In the present article, i*n-silico* studies have been performed to explore the binding modes of Isoxazole substituted 9-aminoacridines(**1a-x**) against SARS CoV-2 main protease (PDB id - 5R82) targeting corona virus using Schrodinger suit 2019-4. The docking studies are performed by Glide module, in-silico ADMET screening was performed by qik prop module and the binding energy of ligands was calculated using PRIME MM-GB/SA module. From the results, Isoxazole substituted 9-aminoacridines like **1n,f,c,k,h,a,e,g,b,d** are significantly active against SARS CoV-2 main protease with Glide score more than -5.5 when compared with currently recommended drug for COVID19 Hydroxy chloroquine (G score -5.47) and Co crystallized ligand CID_24701445 (G score -4.4). The docking results of the compounds exhibited similar mode of interactions with COVID19 and the residues THR24, THR25, THR26, SER46, MET49, HIE41, GLN189, ARG189, ASP187, MET168, HIE164, ASN142 and GLY143 play a crucial role in binding with ligands.

Keywords: SARS CoV-2 main protease, COVID19, Acridine, Isoxazole, Docking studies, *In-silico* ADMET, MMGBSA.

1. INTRODUCTION

Coronavirus Disease 2019 (COVID-19), a life-threatening viral disease which was affected first in Wuhan, china and quickly spread throughout the world[1-5]. According to the data from WHO, as on July 2020, more than 16.5 million peoples in the world affected by COVID19, out of these more than 655,000 peoples are died. With more asymptomatic infections being found among COVID-19 cases, it is worthy of consideration, the detail current evidence and understanding of the transmission of SARS-CoV, MERS-CoV, and SARS-CoV-2 and discuss pathogen inactivation methods on coronaviruses is very important[6-10]. In this emergency situation, it is very important to discover novel drugs for the treatment of COVID19.

9-substituted acridines have reported for different pharmacological activities like anticancer[11-13], antimicrobial[14], antioxidant[15], antimalarial[16], analgesic[17], antileishmanial[18], antinociceptive[19], acetyl cholinesterase inhibitors[20] and antiherpes[21] and so forth. Amsacrine which is 9-anilinoacridine derivative was primary DNA-intercalating agent which is more significant and the chromophore intercalates with DNA base pairs[22]. Likewise, isoxazole derivatives also reported for various biological activities[23-28] like antimicrobial, anti-cancer etc.

As a part of our ongoing research on searching the

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potent biological molecules against various disease by in-silico and wet lab methods[29-39], we have designed some isoxazole substituted 9-aminoacridines (**1a-x**) against SARS CoV-2 main protease (PDB id - 5R82) targeting corona virus using Schrodinger suit 2019-4. Using different modules (Glide, Qikprop and Prime) of Schrödinger suite LLC various computational methods like molecular docking, ADMET screening and binding free energy calculations were performed to find the interactions responsible for SARS CoV-2 main protease inhibition. The outcomes of the research that the recently designed isoxazole substituted 9-aminoacridines showed significant hindrance with COVID19. These studies will provide the requirement of key structural features in the design of potential drug candidates against COVID19.

2. Materials and Methods

The 3D crystal structure of COVID19 protein called SARS-CoV-2 main protease receptor co-crystallized with 6-(ethylamino) pyridine-3-carbonitrile (PDB ID: 5R82, Resolution: 1.31 Å) was retrieved from the RSCB protein data bank[40]. The protein was prepared using protein preparation wizard of epic module[41] of Schrödinger suite 2019-4. The protein structure is a monomer, having similar binding sites were removed by deleting waters, refining bond orders and addition of hydrogens. Missing chains and loops are included by[42] using Prime module of Schrödinger suite 2019-4. Protein energy was minimized by using OPLS3 (Optimized Potentials for Liquid Simulations) molecular force field with RMSD of crystallographic heavy atoms kept at 0.30 Å. A grid box was generated to define the centroid of the active site. All the compounds were docked in to the binding pocket of SARS CoV-2 main protease by using Glide module of Schrödinger suite 2019-4 in XP (Extra precision) mode[43]. To predict the free energy of binding for the ligands in complex with receptor by using Prime Molecular Mechanics-Generalized Born Surface Area (MM-GB/SA) of Schrödinger 2019-4. The energy for minimized XP docked pose of ligand receptor complex was calculated using the OPLS3 force field and generalized-Born/surface area (GB/SA) continuum VSGB 2.0 solvent model[44].

3. Results and discussion

Results are summarized in Table 1-3 and Figure 1-5. The results revealed that the COVID19 inhibitory property of the compounds 1a-x. The Chemical-structures of isoxazole substituted 9-aminoacridines(1a-x) are given in the figure 1.

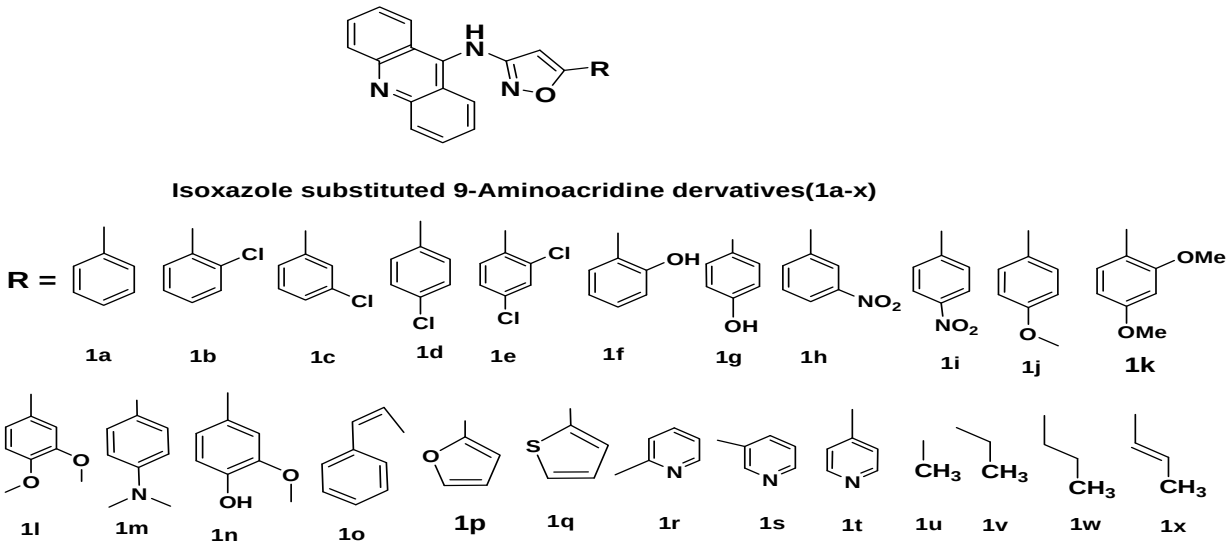

Fig.1. Chemical structures of isoxazole substituted 9-aminoacridines(1a-x)

The docking studies of the ligands to COVID19 protein active sites were performed by the molecular docking program Glide module of Schrodinger suite 2019 Maestro-12.2 version for determining the binding affinities of the compounds. The designed analogues were docked towards the COVID19 (PDB id : 5R82) in order to ascertain their inhibitory activity. The analogues show best fit Root Mean Square Difference (rmsd) value of 0.18. As shown in Table 1, it is clearly demonstrated that Isoxazole substituted 9-aminoacridines like **1n,f,c,k,h,a,e,g,b,d** are significantly active against COVID19 with Glide score more than **-5.5** when compared with currently recommended drug for COVID19 Hydroxy chloroquine (G score -5.47) and co-crystallized ligand (G score -4.4). The above compounds have good affinity to the receptor due to more lipophilic character and also due to hydrogen bonding interactions.

The validation of docking studies are performed by redocking of co-crystallized ligand and compounds. All the ligands are binding in the same active site with same orientation.

The results were summarized in the table **1**. The best affinity modes of all the docked compounds with COVID19 (PDB id: 5R82) were shown in figure **2**. Almost all the compounds are docked in the same binding pocket.

The docking results of the compounds exhibited similar mode of interactions with COVID19 and the residues THR24, THR25, THR26, SER46, MET49, HIE41, GLN189, ARG189, ASP187, MET168, HIE164, ASN142 and GLY143 play a crucial role in binding with ligands.The 2D-ligand interaction diagram of compounds **1n,f,c,k** are given in the figures **3a-d.**

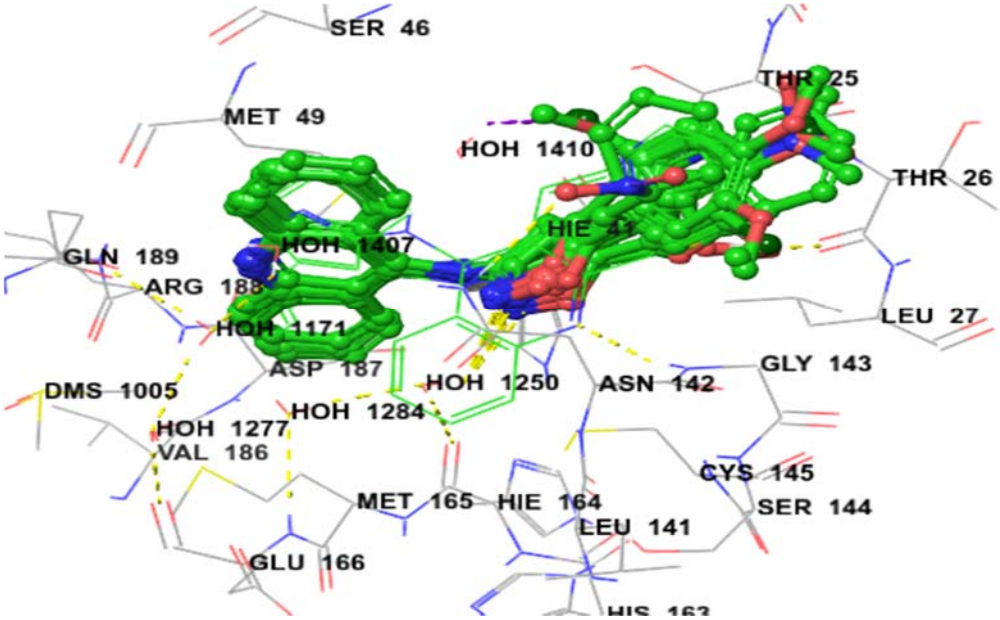

Fig.2.Docked poses of all compounds 1a-x with COVID19 (5R82)

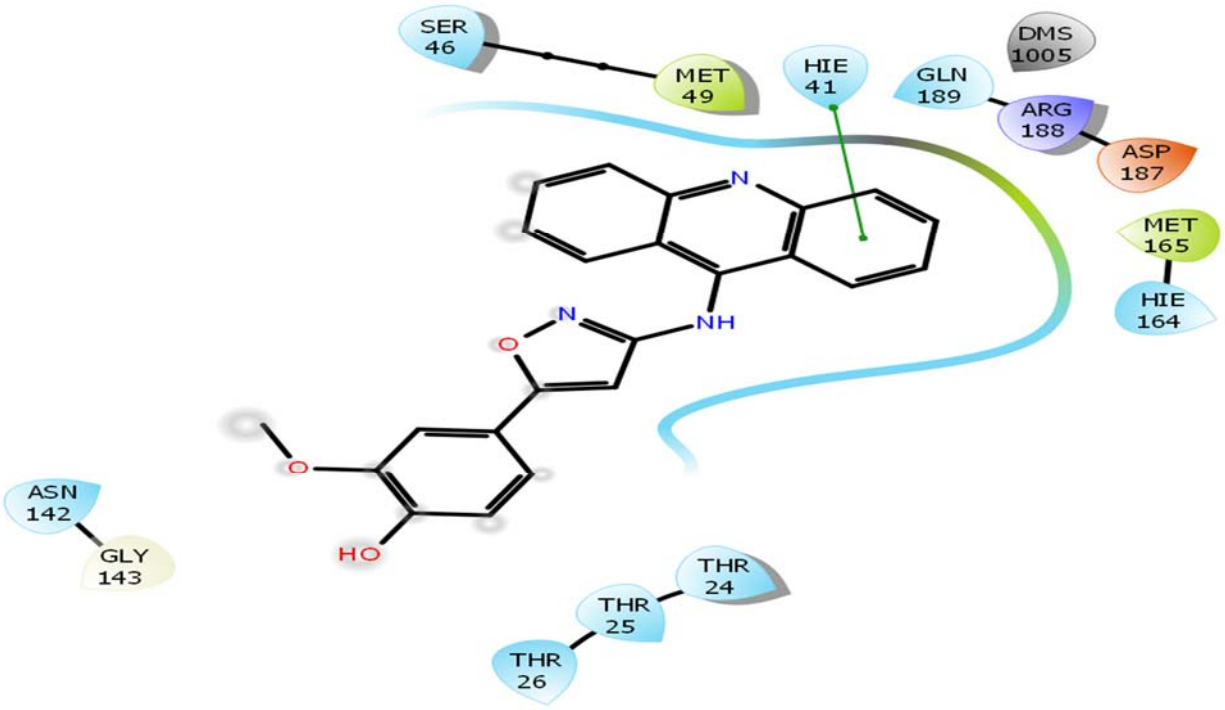

Fig.3.A. Ligand Interaction of compound 1n with COVID19 (5R82)

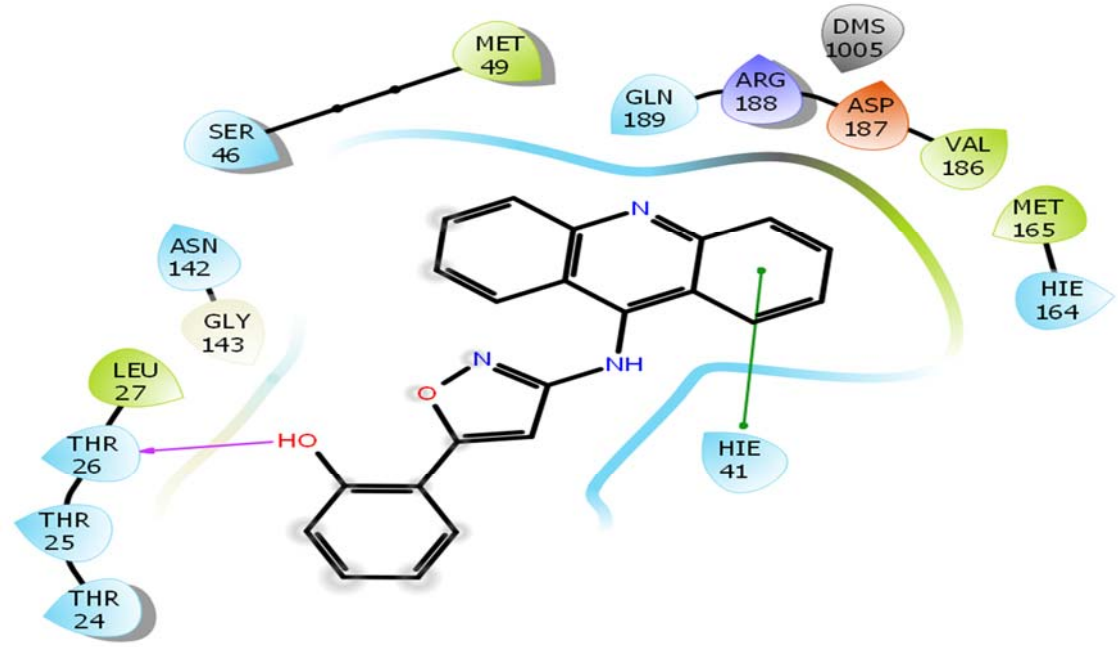

Fig. 3.B. Ligand Interaction of compound 1f with COVID19 (5R82)

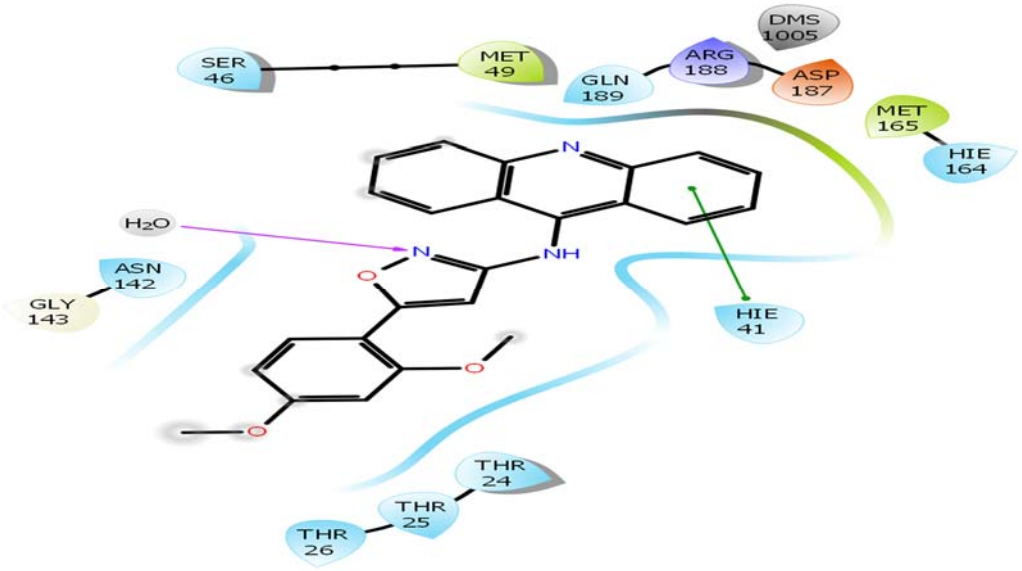

Fig.3.C. Ligand Interaction of compound 1c with COVID19 (5R82)

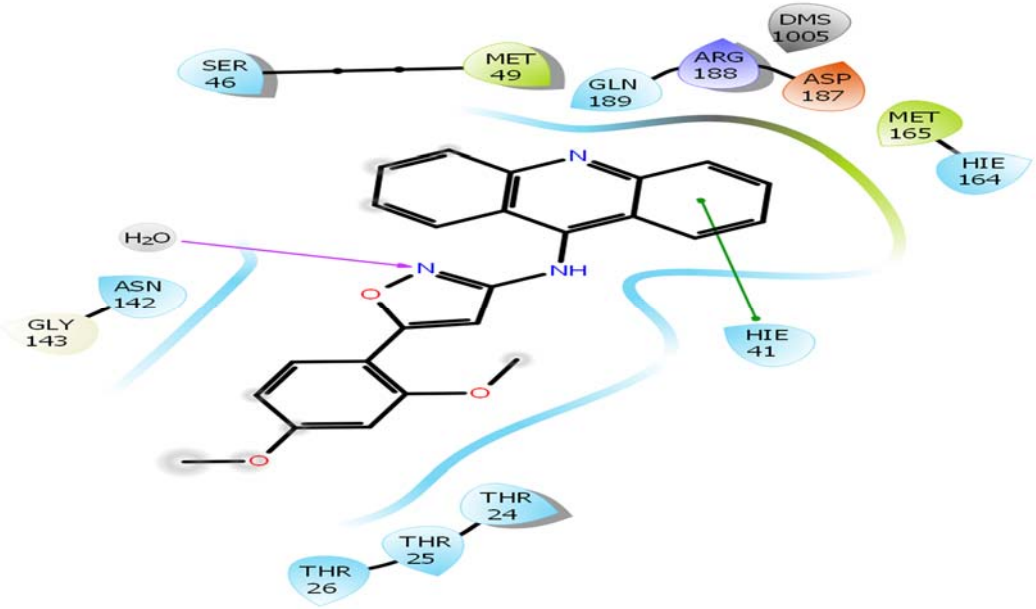

Fig.3.D. Ligand Interaction of compound 1k with COVID19 (5R82)

From the molecular docking study it was revealed that the ligands have shown agreeable glide G score values from -4.64 Kcal/mol (compound 1x) to -6.39 Kcal/mol (compound 1n). From the binding modes obtained, it was illustrated that the ligands **1n,f,c,k,h,a,e,g** formed hydrogen bonding, hydrophobic and other interactions with different residues THR24 to GLN189 surrounding the active pocket which was shown in figure **4**. The ligand**1f**exhibited hydrogen bonding interaction with THR26 (H-Bond length1.75 A°), residue and with some water molecules are shown in the figure **5**. The presence of aromatic features and different heterocyclic rings majorly contributed towards lipophilic factors.

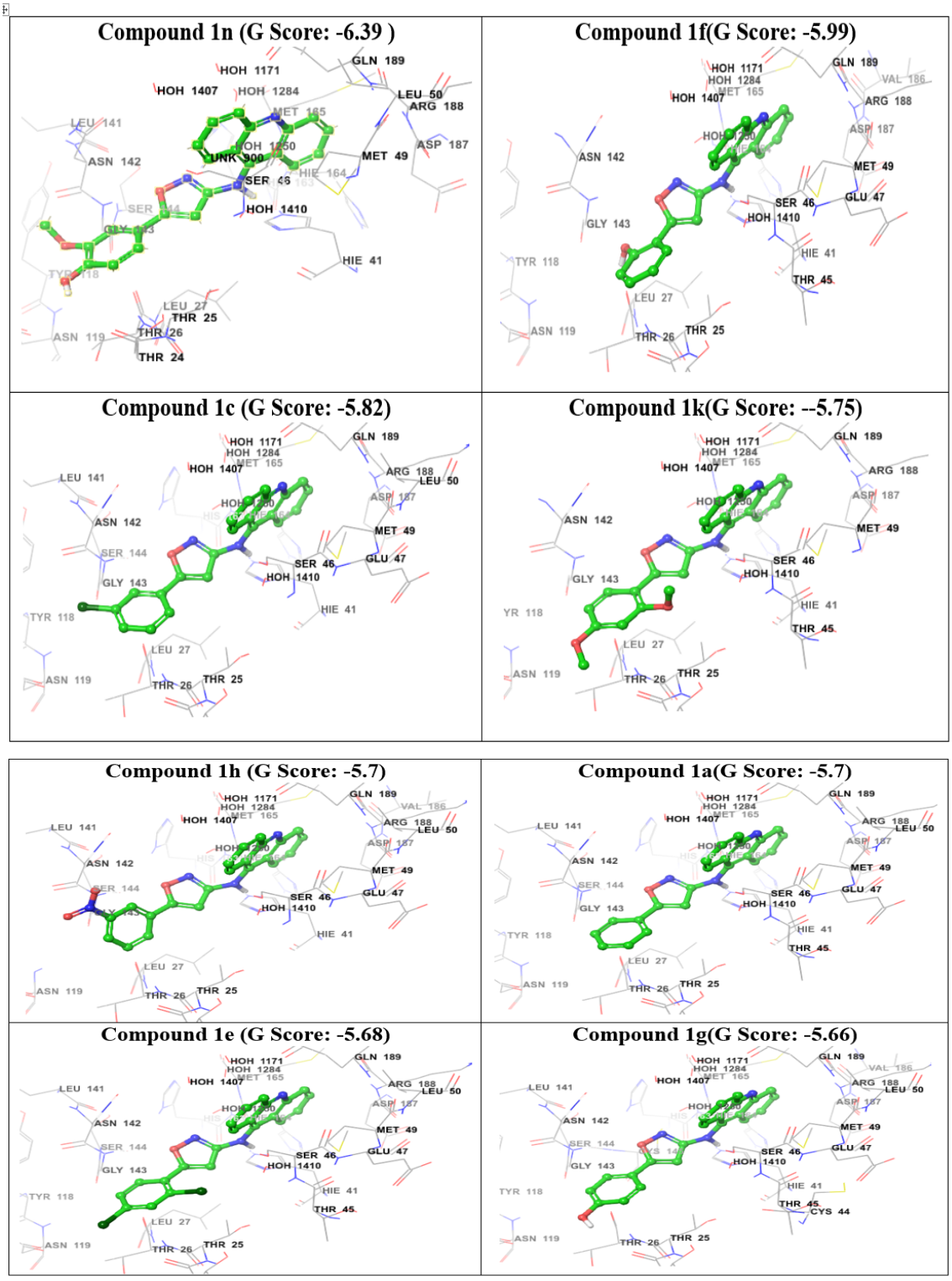

Fig.4. Best affinity mode of docked compounds with COVID19 (5R82)

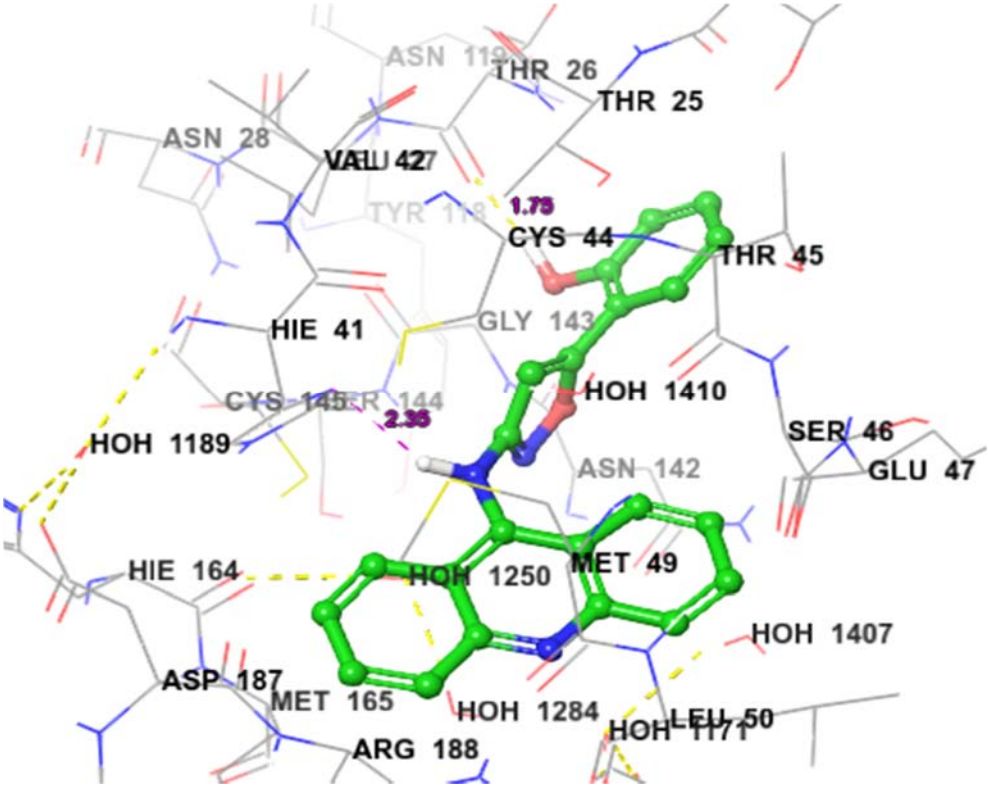

Fig. 5. Hydrogen bonding interaction of compound 1f with COVID19 (5R82)

The ADMET screening for the molecules can be predicted *in-silico* by using qikprop module of Schrödinger suite 2019-4. The results of the ADMET properties for compounds are shown in Table-**2**. From the Table-**2**, *in-silico* ADMET screening results of most the compounds are within the recommended values. The molecular weight between 275.3 and 406.27. Evaluated number of hydrogen bond donors of the molecules are in the range of 1-2. Evaluated number of hydrogen bonds acceptors of most of the compounds are in the range of 3 –5.85. The Prediction of binding to human serum albumin between 0.3 and 1.2. The most of the compounds have QPlogP values are between 3.5 and 5.85. A number of likely metabolites of the compounds are in the range of 0-2. A number of violations of Lipinski's rule of five is 0-1. The compounds have % Human oral absorption in the scope of 94-100%. So from the *in-silico* ADMET screening results of most the compounds are within the recommended values except few parameters of some compounds.

Molecular docking was additionally assessed with MM-GBSA free restricting vitality which is identified with the post scoring approach for COVID19 (PDB ID: 5R82) target and the values are shown in the Table **3**. From the results of MM-GB/SA studies the dG bind values were observed in the range of -22.90 (1h) to -56.797 Kcal/mol (1n) and also dG coulomb, dGvdw values, dG lipophilic values and the energies are positively contributing towards total binding energy. The accuracy of docking is confirmed by examining the lowest energy poses predicted by the scoring function. The Glide score and MM-GBSA free energy are obtained by the docking of ligands into the coupling pocket are more stable.

4. Conclusion

From the results of docking study that the isoxazole substituted 9-aminoacridines like 1n,f,c,k,h,a,e,g,b,d demonstrated better arrangement at dynamic site of the COVID19 protein. The in-silico structuring strategy embraced in the present investigation helped for recognizing some lead molecules such as 1n,f,c,k,h,a,e,g,b,d and furthermore may somewhat clarify their useful impact for the further determinations like in vitro and in vivo assessments. Results from the in-silico study, revealed that many of the isoxazole substituted 9-aminoacridines like 1n,f,c,k,h,a,e,g,b,dmay be useful against COVID19 and are probably going to be helpful after further refinement.

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Table 1. Docking studies for Isoxazole substituted 9-aminoacridines(1a-x) with COVID19 (5R82)

Cpd	Glide score	Lipophilic EvdW	H Bond	XP Electro	Low MW	XP Penalties	Rot Penal
1n	-6.39	-5.65	-0.78	-0.09	-0.22	0	0.17
1f	-5.99	-5.13	-1.23	-0.49	-0.32	0	0.2
1c	-5.82	-5.57	-0.28	-0.08	-0.26	0	0.18
1k	-5.75	-5.56	-0.24	-0.09	-0.18	0	0.16
1h	-5.7	-5	-0.53	-0.17	-0.23	0	0.17
1a	-5.7	-5.21	-0.38	-0.11	-0.38	0	0.21
1e	-5.68	-5.48	-0.22	0.01	-0.15	0	0.15
1g	-5.66	-5.38	-0.37	-0.17	-0.32	0	0.2
1b	-5.54	-5.32	-0.24	-0.05	-0.26	0	0.18
1d	-5.5	-5.32	-0.22	-0.02	-0.26	0	0.18
1i	-5.49	-5.28	-0.29	-0.02	-0.23	0	0.17
1w	-5.47	-4.82	-0.46	-0.1	-0.49	0	0.26
1q	-5.47	-5.08	-0.28	-0.13	-0.36	0	0.21
1j	-5.46	-5.33	-0.24	-0.04	-0.28	0	0.18
1p	-5.46	-5	-0.37	-0.15	-0.41	0	0.23
1v	-5.26	-4.54	-0.41	-0.13	-0.5	0	0.19
1m	-5.21	-5.6	-0.22	-0.09	-0.23	0	0.17
1t	-5.15	-5.13	-0.17	-0.05	-0.37	0	0.21
1s	-5.12	-5.28	-0.66	-0.11	-0.37	0	0.21
1l	-4.96	-5.12	0	-0.1	-0.18	0	0.16
1u	-4.95	-4.46	-0.3	-0.06	-0.5	0	0.2
1r	-4.87	-4.75	-0.43	-0.14	-0.37	0	0.21
1o	-4.81	-5.24	-0.52	-0.18	-0.29	0	0.25
1x	-4.64	-3.51	-0.7	-0.2	-0.5	0	0.26
Hydroxy chloroquine (Std)	-5.47	-3.15	-1.75	-0.69	-0.38	0.5	0
CID_24701445_Co-ligand	-4.4	-2.88	-0.7	-0.21	0.5	0.29	0.29

Table 2. *In-silico* ADMET screening for Isoxazole substituted 9-aminoacridines

Compounds	Mol. Wt.	Donor HB	Accpt HB	QPlogKhsa	QPlog o/w	#metab	Rule of Five	%Human Oral Absorption
1a	337.38	1	3	0.869	4.88	0	0	100
1b	371.825	1	3	0.936	5.124	0	1	100
1c	371.825	1	3	0.991	5.378	0	1	100
1d	371.825	1	3	0.989	5.377	0	1	100
1e	406.27	1	3	1.099	5.848	0	1	100
1f	353.379	2	3.75	0.641	4.224	1	0	100
1g	353.379	2	3.75	0.662	4.094	1	0	100
1h	382.378	1	4	0.826	4.182	1	0	94.046
1i	382.378	1	4	0.83	4.194	1	0	94.091
1j	367.406	1	3.75	0.886	4.99	1	0	100
1k	397.432	1	4.5	0.869	5.008	2	1	100
1l	397.432	1	4.5	0.92	5.159	2	1	100
1m	380.448	1	4	1.037	5.303	1	1	100
1n	383.406	2	4.5	0.68	4.339	2	0	100
1o	363.418	1	3	1.155	5.884	0	1	100
1p	327.342	1	3.5	0.604	4.23	1	0	100
1q	343.402	1	3	0.794	4.79	1	0	100
1r	338.368	1	4	0.647	4.229	1	0	100
1s	338.368	1	4.5	0.541	3.944	2	0	100
1t	338.368	1	4.5	0.542	3.944	2	0	100
1u	275.309	1	3	0.398	3.505	1	0	100
1v	289.336	1	3	0.506	3.847	1	0	100
1w	303.363	1	3	0.627	4.231	1	0	100
1x	301.347	1	3	0.627	4.17	1	0	100
Hydroxy chloroquine(std)	335.876	2	5.7	1.085	3.369	5	0	93.213
Recommended values	130-725	0– 6	2-20	-2-8.5	-2-6.5	1 – 8	max 4	>80% is high <25% is poor

MW- Molecular weight of the molecule,

donorHB - Estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution.

accptHB- Estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution

QPlogKhsa- Prediction of binding to human serum albumin.

QPlogPo/w - Predicted octanol/water partition coefficient.

#metab- Number of likely metabolic reactions.

RuleOfFiveNumber of violations of Lipinski's rule of five.

%Human- Oral absorption- Predicted human oral absorption on 0 to 100% scale.

Table 3. Binding free energy calculation using Prime/MM-GBSA approach

Compd	MMGBSA_ dG_Bind	MMGBSA _dG_Bind_ Coulomb	MMGBSA _dG_Bind_ Covalent	MMGBSA_dG_Bind Hbond	MMGBSA _dG_Bind_ Lipo	MMGBSA_dG_Bind_ vdW
1n	-56.7968	-23.5154	2.4804	-1.8585	-10.9638	-40.0530
1f	-53.4693	-36.973	10.0153	-3.5311	-15.3509	-34.2833
1c	-47.3019	-11.948	10.2109	-1.0722	-13.2933	-45.2213
1k	-43.9099	-15.814	2.2821	0.3468	-13.1878	-40.3116
1h	-22.8992	6.9013	-2.0890	1.7164	-11.3424	-31.7120
1a	-49.1943	-30.712	5.8477	-1.7633	-13.6913	-31.6009
1e	-42.2462	-25.773	-0.4271	0.5160	-10.2413	-34.5282
1g	-46.7504	-36.282	4.1380	-0.6984	-11.9709	-27.1283
1b	-50.9684	-32.433	5.3735	-0.8809	-14.1885	-32.4094
1d	-47.7638	-32.581	9.3811	-1.57074	-10.6278	-30.9074
1i	-41.5192	-16.310	7.1774	0.1748	-11.0601	-41.2056
1w	-18.7796	-4.4731	0.8444	1.1242	-7.2195	-33.4287
1q	-44.1846	0.6761	-2.0777	1.1035	-11.8353	-42.0376
1j	-30.3496	-24.485	4.6831	0.2354	-12.5731	-33.7760
1p	-41.1950	-12.571	1.4255	0.1776	-11.0097	-36.9079
1v	-38.7516	-6.2809	4.3009	-0.8058	-10.4623	-35.3600
1m	-37.5011	-24.428	3.8591	0.2568	-9.5163	-34.8443
1t	-43.1380	-35.158	10.6070	-1.0321	-10.5935	-31.661
1s	-50.5496	-27.102	17.2451	-1.0286	-17.0153	-41.0016
1l	-46.3071	-17.765	-0.1487	1.0456	-9.0579	-44.2125
1u	-37.9464	-14.893	1.4122	-1.1176	-6.2972	-30.8720
1r	-39.5155	-18.112	0.2818	-0.0504	-11.9452	-33.9023
1o	-38.3410	-14.036	10.5528	-0.1116	-11.5826	-40.0713
1x	-39.1372	-1.2618	11.6664	-0.1053	-15.1498	-34.6229
Hydroxy Chloroquine (std)	-26.9975	-4.9621	2.1824	0.0011	-9.2894	-33.0622

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نشاط Isoxazole استبدال Aminoacridines−9 ضد السارس CoV−2 بروتياز الرئيسية ل COVID19: نهج حسابي

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ملخص

مرض فيروس كورونا 2019 (COVID-19)، وهو مرض فيروسي مهدد للحياة يتأثر أولا في الصين وينتشر بسرعة في جميع أنحاء العالم in في أوائل عام2020. الكثير من cientists يسارعون لاكتشاف الأدوية واللقاحات الجديدة ضد الفيروس التاجي ، والعلاجات ل COVID - 19. أنان هذه المادة، وقدأجريت دراسات ط نسيليكو لاستكشاف طرق ملزمة من Isoxazole استبدال aminoacridines (1a-x−9) ضد السارس CoV-2 protease الرئيسية (PDB id - 5R82) استهداف فيروس كورونا باستخدام دعوى Schrodinger 2019-4. يتم إجراء دراسات الإرساء بواسطة وحدة Glide ، وتم إجراء فحص ADMET في silico بواسطة وحدة دعامة qik وتم حساب الطاقة الملزمة لليغند باستخدام وحدة PRIME MM-GB / SA. من النتائج، Isoxazole استبدال aminoacridines−9 مثل n1، f، c، k، h، a،e،g،b،d نشطة بشكل ملحوظ ضد سارس CoV-2 protease الرئيسية مع الإنزلاق درجة أكثر من −−5.5 بالمقارنة مع المخدرات الموصى بها حاليا ل COVID19 هيدروكسي الكلوروكين (G النتيجة −5.47) وشركاه بلورة ليغاند G) CID_24701445 النتيجة −4.4). أظهرت نتائج الالتحام للمركبات طريقة مماثلة من التفاعلات مع COVID19 والمخلفات THR24، THR25، THR26، SER46، MET49، HIE41، GLN189، ARG189، ASP187، MET168، HIE164، ASN142 و GLY143 تلعب دورا حاسما في الربط مع ليغاندس.

الكلمات الدالة: سارس (CoV−2)،(COVID19)

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Comparative study of selected *Rosa* varieties' metabolites through UPLC-ESI-MS/MS, chemometrics and investigation of their insecticidal activity against *Culex pipiens* L.

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ABSTRACT

The 70 % methanolic extracts of three *Rosa* varieties (aerial parts & flowers); *Rosa banksiae* var. *banksiae* Ait. (RBW); *Rosa polyantha* Thunb. 'orange fairy' (RPO) and *Rosa polyantha* Thunb. 'white fairy' (RPW) were quantified using UPLC-ESI-MS/MS. Sixty one compounds were identified where flavonoid glycosides were the most abundant. Principal component analysis (PCA) and Hierarchical cluster analysis (HCA) discriminated the six samples into four clusters where RBW-F & RPW-A formed one cluster, RPW-F & RPO-F formed another and RPO-A and RBW-A were in two separate clusters. The six *Rosa* extracts were tested as insecticidal agents against *Culex pipiens* L. larvae and pupae and their fecundity reducing activity was evaluated for the emerging adult mosquitoes. RPW flowers and aerial parts' extracts demonstrated powerful larvicidal and pupicidal activities with LC_{50} 373.3 and 383.2 ppm, respectively. Sterility indices reached 51.4% at highest concentrations used. All flower extracts possessed significantly high mortality and sterility activities ($P < 0.001$) compared to the aerial parts ($P < 0.01$).The UPLC-MS/MS metabolic profile of the extracts showed their richness in polyphenolic compounds. The 70% methanolic extracts of RBW, RPO and RPW aerial parts and flowers can be utilized as natural and safe plant-based insecticides for *C. pipiens* (filariasis vector) control.

Keywords: *Rosa*, UPLC-MS/MS, metabolomics, *Culex pipiens*, filariasis.

INTRODUCTION

Genus *Rosa* is among the largest genera belonging to family Rosaceae with more than 250 species mostly located in China and South-West Asia. *Rosa banksiae var. banksiae* Ait. is known as bankasian rose or lady banks, after Sir Joseph Banks's wife, usually seen as part of parks decoration and as ornamental flower in tombstones [1]. Bankasian rose is a climber shrub with white, yellow and rose colored flowers which blooms heavily during Spring in fascinating way. Polyantha flowers are part of modern roses, they are named *R. polyantha* Thunb. (*R. multiflora* Thunb.) due to their heavy flowering nature and the fairy roses are a new hybrid roses from this class since 1930s [2].

Family Rosaceae is rich in flavonoids [3], terpenoids [4], tannins [5] and fatty acids [6]. Most of the previous secondary metabolites studies were done using targeted approaches, focusing on certain class of compounds [7]. Nowadays, metabolomics play a pivotal role in the identification and quantification of a large set of metabolites through their molecular ion peaks and characteristic fragmentation pattern, taking advantage of the development of hyphenated techniques *viz.* tandem mass spectrometry (LC-MS/MS) [8]. Nevertheless, it has the advantage of saving time, money and efforts in isolating the secondary metabolites [9].

Early reports showed that essential oil of *R.*

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damascena Mill exerted a powerful contact, repellent and ovicidal activity against certain spider [10]. Plant derived insecticides have emerged as safe, affordable and effective alternative agents for mosquito vector control [11]. They are mostly target specific and readily biodegradable compared to chemical counterparties. The first known bio-insecticide was isolated from pyrethrum affected the insect nervous system. Regarding chemical insecticides, they are rapid, effective and broad spectrum killing agents, but they carry a lot of drawbacks being toxic to non-target organisms like fishes leading to direct and indirect toxic effects to the environment and human, as well as being highly resisted by many mosquito disease vectors [11].

Culex mosquito is the main vector transmitting *Wuchereria bancrofti* parasite which causes human lymphatic filariasis which is considered one of the neglected tropical diseases (NTDs). World health organization (WHO) has launched a worldwide program in 2000 with a goal of eliminating filariasis by 2020 which will never be possible unless immense efforts are to be done in order to control the main transmitting vector side by side to other management strategies [12].

The aim of this study was to unravel the metabolome composition of some selected species of genus *Rosa* cultivated in Egypt using ultra performance liquid chromatography – electrospray ionization - tandem mass spectrometry (UPLC-ESI-MS/MS) coupled to chemometrics *viz.* PCA and HCA for differentiating between three *Rosa* varieties with two different organs and evaluate their insecticidal potency against filariasis vector *Culex*; thus providing a safe natural source of plant derived insecticide to combat one of highly dangerous diseases in Africa.

Results

1. Identification of *Rosa* species metabolites through tandem mass spectrometry

Ultra performance liquid chromatography - tandem mass spectrometry (LC-MS/MS) has been approved for long as standard analysis and authentication tool for characterization and identification of plant metabolomics and profiling of different plant active constituents. Moreover, LC-MS/MS plays a fundamental role in the quantification of different constituents; compounds identification and confirmation relied upon both retention time and mass fragmentation [13]. Sixty one compounds, were tentatively identified and quantified in Table A. 1, Figures A. 1 and A. 2 and arranged in descending manner according to their retention times. The identified compounds can be classified into: 30 flavonoids, 12 phenolic acids, 8 tannins, 5 terpenoids, 2 alkaloids, 2 cyclic polyols, 1 anthocyanin and 1 chromone (Figure A. 3).

The highest number of identified compounds (Table A.1) were in RBW-A (51 compounds) followed by RPW-A (29 compounds) and RBW-F (26 compounds). The percentage identification ranged *ca.*70.41 % to 96.49% in ESI negative mode and *ca.* 16.70% to 43.99% in ESI positive mode. This confirmed that the negative mode can be used better for identification of polyphenolic compounds.

For the aerial parts, RBW-A was rich in chlorogenic acid **(M65)**, 20.96%; apigenin-6-*C*-pentoside **(M39)**, 15.75% and quercetin-7-*O*-hexoside-3-*O*-(malonyl)-hexoside **(M27)**, 10.97%. For RPO-A, quinic acid **(M1)**, 70.11%; chlorogenic acid **(M65)**, 18.24% and pibtocarphin B **(M74)**,10.95% were among the abundant compounds, while for RPW-A, they were chlorogenic acid **(M65)**, 16.89%;quinic acid **(M1)**, 15.28%; quercetin-3-*O*-glucouronide **(M17)**, 13.85% and quercetin aglycone **(M18)**, 13.55%.

The flower extracts major identified compounds were; quinic acid **(M1)**, 17.13%; chlorogenic acid **(M65)**, 13.80%; kaempferol-3-*O*-glucouronide **(M22)**, 11.95% and quercetin-7-*O*-hexoside-3-*O*-(malonyl)-hexoside **(M27)**, 11.79% for RBW-F; quinic acid **(M1)**, 26.03%, kaempferol-3-*O*-rhamnoside **(M26)**, 24.69%; gallic acid **(M4)**, 15.02% and quercetin-3-*O*-rhamnoside **(M21)**, 13.44% for RPO-F and kaempferol-3-*O*-rhamnoside **(M26)**, 25.29%, quinic acid **(M1)**, 17.88% and quercetin-3-*O*-rhamnoside **(M21)**, 13.31% for RPW-F (Figure A. 3).

1.1. Flavonoid glycosides and their derivatives

The studied *Rosa* species herein were heavily enriched with quercetin and kaempferol either as aglycones or different forms of glycosides (Table A. 2) and detailed as

follows; compound **(M14)** with $[M-H]^-$ *m/z* 433 and $[M+H]^+$ *m/z* 435 was defined as quercetin-3-*O*-arabinoside and it gave daughter molecular fragment at *m/z* 301 for quercetin aglycone [14]. Another quercetin pentoside was detected at $[M-H]^-$*m/z* 447 and $[M+H]^+$ *m/z* 449 and was tentatively assigned to quercetin-3-*O*-rhamnoside **(M21)**. Similarly, quercetin-3-*O*-glucouronide **(M17)** was identified at $[M-H]^-$ *m/z* 477 with one major fragment at *m/z* 301 for quercetin aglycone. In both ESI positive and negative ion modes, quercetin aglycone **(M18)** was clear at *m/z* 301 and 303, respectively [15]. A set of compounds showing same $[M-H]^-$ *m/z* 711 were detected at different retention times as so were considered sugar isomers and tentatively defined as quercetin-7-*O*-hexoside-3-*O*-(malonyl) hexoside **(M27)** and its two isomers **(M30)** and **(M34)**[16]. Two commonly found compounds in citrus fruits were seen at $[M-H]^-$ *m/z* 757 and they were tentatively identified as eriodictyl-4'-*O*-neohesperidoside-7-*O*-glucoside **(M8)** with its daughter moieties at *m/z* 595 and 449 due to loss of hexoside moiety as glucose and neohesperidoside group, respectively and isorhamnetin-3-*O*-rutinoside **(M12)** at $[M-H]^-$ *m/z* 623[15].

On the other hand, kaempferol aglycone **(M32)** was detected in ESI –ve mode at *m/z* 285 and at *m/z* 287 in ESI +ve mode, while kaempferol-3-*O*-glucouronide **(M22)** was identified at $[M-H]^-$ *m/z* 461 [14] and it was only found in RBW-F. Another glucouronide was shown at $[M-H]^-$ *m/z* 593 and at $[M+H+Na]^+$ *m/z* 617 was defined as kaempferol-*O*-pentose-*O*-glucouronic acid **(M25)** with its fragments at *m/z* 441 and 417 for the successive loss of pentose and glucouronic acid, respectively. A deprotonated molecular ion was detected at $[M-H]^-$ *m/z* 417 and $[M+H+Na]^+$ *m/z* 441 and was agreed to be kaempferol-3-*O*-arabinoside **(M24)**. With rhamnose sugar moiety, kaempferol-3-*O*-rhamnoside **(M26)** was identified with $[M-H]^-$ *m/z* 431 and $[M+H+Na]^+$ *m/z* 455 [17]. Compound **(M29)**, identified as kaempferol-3-*O*-rutinoside showed molecular ion peak also at *m/z* 593 only in negative mode but with one fragment at *m/z* 285 for kaempferol aglycone [14].

Apigenin-6-*C*-pentoside **(M39)** was traced at $[M-H]^-$ *m/z* 503 and $[M+H+Na]^+$ *m/z* 527 [18]. Naringenin aglycone **(M63)** was found at *m/z* 271 in negative mode [15] with traces of its galloylated, acetylated and methylated derivatives such as quercetin-*O*-(2"-*O*-galloyl)-hexoside **(M13)** and Kaempferol-galloylhexoside **(M19)** which were identified in negative mode at $[M-H]^-$ *m/z* 615 and *m/z* 599, respectively. Another galloylated flavonoid glycoside was traced at $[M-H]^-$ *m/z* 601 which was recognized as myricetin-*O*-(*O*-galloyl)-pentoside **(M69)** and was further confirmed by a fragment at *m/z* 449 for the loss of pentose sugar. One acetylated flavonoid glycoside was assigned to (iso) rhamnetin-3-*O*-6"-*O*-acetyl-glucoside **(M36)** with a deprotonated peak at *m/z* 519 in negative mode [14]. 7-*O*-methylated flavonoid was detected at $[M+H]^+$ *m/z* 301 for compound **(M52)** which was identified tentatively as rhamnocitrin.

1.2. Phenolic Acids

Electrospray ionization negative ion mode revealed a deprotonated molecular ion peak for gallic acid **(M4)** at $[M-H]^-$ *m/z* 169 [14], gallic acid was traced only in RBW-F and RPO-F, it was also identified in other *Rosa* species[19]. Different caffeic acid derivatives were quantified (Table A. 2) where a molecular ion peak was shown at $[M-H]^-$ *m/z* 487 in negative mode and $[M+H+Na]^+$ *m/z* 511 in positive mode and it was assigned to caffeoylhexose deoxyhexoside **(M48)** with two major fragments at *m/z* 308 and 179 for loss of caffeoyl and deoxy hexose hexoside moieties, respectively. Compounds **(M49)** and **(M54)** were at the same molecular ion peak as compound **(M48)** which was attributed to presence of isomerism in the hexose sugar moiety so they appeared at different retention times with the same *m/z* value and fragmentation pattern. A dimer form of caffeic acid was detected at $[M-H]^-$ *m/z* 683 which was identified as caffeic acid-*O*-hexoside dimer **(M85)** and its identity was confirmed by its fragment ions at *m/z* 341 and 179 for the splitting of the dimer molecule to two monomers and

the loss of hexose molecule, respectively. In addition to that, an *O*-methylated phenolic acid known as syringic acid **(M89)** was detected at $[M-H]^-$ *m/z* 197. Another phenolic acid was identified as *p*-coumaric acid hexoside **(M11)** at *m/z* 325 in negative mode [18]. It is worthy noted that, the bankasian rose aerial parts were rich by caffeic and ferulic acid derivatives while gallic acid was not traced in it. Three cinnamic acid derivatives were observed namely; 5-*O*-*p*-coumaroyl-4-*O*-caffeoyl-4-methylpentanoic acid-5-hydroxy-3-quinate **(M37)**, chlorogenic acid (caffeoylquinic acid) **(M65)** and its isomer **(M67)** at $[M-H]^-$ *m/z* 693 and $[M+H]^+$ *m/z* 353, respectively.

1.3. Terpenoids

A lanostane type triterpenoid was spotted at $[M-H]^-$ *m/z* 501 for ganolucidic acid B **(M42)** together with daughter peaks at *m/z* 483, 394 and 377 due to $[M-H-OH]^-$, $[M-H-C_5H_6O_3]^-$, $[M-H-C_6H_6O_3]^-$ fragments [20]. Alisol C **(M47)**, a protostane type triterpenoid, was discovered at *m/z* 485 in negative mode with one fragment at *m/z* 468 due to loss of hydroxy group [21]. Oleanolic acid **(M61)** and its isomer, ursolic acid **(M64)**, were tentatively identified at the same $[M-H]^-$ *m/z* 455 due to isomerism but at different retention times [22]. Only one sesquiterpenoid named piptocarphin B **(M74)** was distinguished at $[M+H]^+$ *m/z* 437 and its side chain fragment appeared at *m/z* 169 [23].

1.4. Tannins

Different forms of hydrolysable tannins had been tentatively determined such as hexahydroxydiphenic acid hexoside **(M2)** which appeared at $[M-H]^-$ *m/z* 481, galloylquinic acid **(M3)** at *m/z* 343 deprotonated molecular ion peak in negative mode with fragmentation peak at *m/z* 191 for quinic acid moiety [18]. At $[M-H]^-$ *m/z* 633, HHDP-galloylglucopyranoside **(M6)** was defined and confirmed by HHDP and quinic acid moieties at *m/z* 301 and 191, respectively. A trigalloyl hexose **(M28)** was pointed at $[M-H]^-$ *m/z* 635 [24] as well as digalloylhexose tannin **(M60)** at *m/z* 483 in negative mode with fragment at *m/z* 331 due to loss of hexoside. Condensed tannins were less represented; 3-methyl-epigallocatechin gallate and its isomer were shown at $[M-H]^-$ *m/z* 471 as compounds **(M56 & M57)** with fragment at *m/z* 269 due to breaking of methyl gallate moiety. A fragment of proanthocyanidin **(M23)** was found at $[M+H]^+$ *m/z* 287 [25] .

1.5. Anthocyanins

Compound **(M77)** with $[M+H]^+$ *m/z* 503 and MS/MS fragments at *m/z* 327, 281, 265, 249 and 205 was identified as pelargonidin-succinyl-arabinoside or pelargonidin-malonyl rhamnoside [25].

1.6. Alkaloids

Ergocristine **(M78)** and its dehydrate form **(M87)** were detected at $[M+H]^+$ *m/z* 610 and 592 [26], respectively with daughter fragments at *m/z* (436, 221, 43) and (330, 289, 237, 69), respectively.

1.7. Miscellaneous

A deprotonated molecular ion peak was traced at $[M-H]^-$ with *m/z* 191 in negative mode representing quinic acid **(M1)**, with daughter fragment at *m/z* 145 due to loss of terminal COOH group [18]. Another peak appeared **(M66)** at *m/z* 279 in negative mode for quinic acid derivative [27].

2. Chemometrics analysis

Metabolic profiling (61 components, Table A. 1) were subjected to both PCA and HCA to reveal the chemical variability, and the inter-relationships between the extracts of the three studied *Rosa* varieties (aerial parts and flowers).

PCA score plot explained 79% of the variance of the data for the three varieties *viz.* RBW, RPO, RPW aerial and flowers, as shown in Figure A. 4 (A & B). Four clusters had been constructed where RPO-A cluster lies in the lower right quadrant; RPW-F and RPO-F lie in the upper left quadrant. Nevertheless, RPW-A and RBW-F were superimposed over each other in the left lower quadrant together with RBW-A. Loading plots Figure A. 4B, showed that the main discriminating marker was quinic acid for RPO-A. However, quercetin-7-*O*-hexoside-3-*O*-(malonyl)-hexoside was the leading metabolite discriminating RPW-F and RPO-F. Loading

plots displayed ganolucidic acid as the main discriminating markers for RBW-F and RPW-A, while trigalloyl hexose and tricin aglycone were specific to RBW-A. Additionally, HCA was applied as unsupervised pattern recognition method in order to confirm results obtained by PCA. The dendrograms obtained for all *Rosa* varieties endorsed the results of PCA as shown in Figure A. 4. The main identified compounds in the six samples were represented in clustered heatmap (Figure A. 6) where the highest concentrations were in red and the lowest in blue shades and between them lies areas in yellow and green for medium concentrations.

3. Insecticidal activity of *Rosa* extracts against *Culex pipiens*

3.1. Effect of different *Rosa* extracts on different stages of *Cx. pipiens*

The biological activity of the tested RBW, RPO and RPW extracts varied according to plant part used and the concentration of the extracts. Both larval and pupal mortality percentages were increased linearly by increasing the concentration of the tested extracts. Flower extracts were more effective against *Cx. pipiens* larvae and pupae than the aerial parts for all *Rosa* extracts (Table A.2). Complete larval mortality percent (100.0%) occurred at the highest concentration (700ppm) of RPW aerial parts and flowers. Also, the highest pupal mortality percent (38.9%) was recorded by RPW-A extract at 600ppm, respectively. According to LC_{50} values for different flower extracts , RPW-F (373.3ppm) was the most potent followed by RBW-F (479.3ppm) and RPO-F (554.1ppm), while the LC_{50} values for aerial parts were 383.2, 491.4 and 862.3ppm for RPW, RPO and RBW, respectively (Figure A. 5).

Regarding the larval and pupal periods, all tested extracts prolonged these periods at the highest concentrations significantly ($P<0.05$), while RBW-F insignificantly ($P>0.05$) prolonged larval and pupal periods at all concentrations used as compared with untreated group (Table A. 2).

3.2. Effect of *Rosa* extracts on the reproductive potential of resulted females

RBW, RPO and RPW extracts significantly ($P<0.001$) reduced the fecundity and increased the sterility % of females developed from treated larvae as compared with the untreated groups, the fecundity and sterility percentages were varied according to plant part and concentration of the extract (Table A. 3). In addition, a noticeable decrease in the hatchability % of eggs laid by females resulted from treated larvae with *Rosa* extracts was observed in a dose dependent manner. RPW aerial parts and flowers extracts recorded the highest sterility index percentages (56.1 and 55.5) at the same concentration (600ppm), respectively, compared with 10.4 and 9.1% sterility in the untreated groups.

Discussion

LC-ESI-MS/MS plays fundamental role in the metabolomic profiling of plant extracts. Different plant extracts can be compared side by side both qualitatively and quantitatively through LC-MS/MS analysis. Different components can be identified and quantified with their daughter fragments which helps in profiling and fingerprinting plant species and varieties with close chemical composition. Herein three varieties, *Rosa banksiae var. banksiae* Ait., *R. polyantha* Thunb. 'orange fairy' and *R. polyantha* Thunb. 'white fairy' belonging to genus *Rosa* were analysed through tandem mass and their components were revealed and compared.Flavonoids and their derivatives are the most abundant class of phytoconstituents identified through LC-ESI-MS/MS which is in accordance with most of the other studied *Rosa* extracts. Different studies utilized LC-MS analysis for the analysis of different *Rosa* species where phenolic acids, flavonols and anthocyanins were identified from the rosehips extract of *R. canina* [28]. Phenolic acids, flavonols and hydrolysable tannins were the main components of *R. rugosa* petal extract analysed using UPLC-PDA-Q/TOF-MS [29]. The stem extracts of *R. moschata*, *R. canina* and *R. sempervirens* were compared through LC-ESI-

MS and flavonoids were abundant in the three of them [30]. Flavonoids and their aglycones were the major classes identified from *R. rugosa* leaves and achenes [19].

Principal component analysis (PCA) and Hierarchical clustering analysis (HCA) chemometric tools further discriminated the metabolomics data arising from the LC-MS/MS analysis resulting in four main clusters that were separated according to their spatial composition with one or two discriminating component for each cluster. The main identified compounds were colour coded in the clustered heatmap in which they were arranged in line with the PCA and HCA results. Here chemometrics played added role in comparing and differentiating the six *Rosa* samples and helped in their differentiation by showing one or more main differentiating component for each.

The obvious existence of flavonoids in the three *Rosa* varieties had played an important role in their insecticidal activity *viz.* larvicidal, pupicidal and fecundity activities [31]. The flower extracts were more potent as an insecticide than the aerial parts extracts. *Rosa polyantha* Thunb. white fairy (RPW) aerial parts and flower extracts showed the strongest larvicidal, pupicidal and fecundity activities against *Cx. pipiens* compared to others. Flavonoids had previously proven their role in regulating fecundity and feeding activities of insects exposed to them [32] *e.g.* quercetin-3-*O*-rutinoside showed oviposition deterrence against cabbage butterflies[33]. Different quercetin and kaempferol glycosides identified form *Kalanchoe beharensis* and *K. longiflora* leaves showed their strong insecticidal activity for the cotton worm [34]. Plant flavonoids usually provoke their insecticidal activity through their neuroactivity on the insects' central nervous system by inhibition of acetylcholine esterase [32].

General Experimental

1. Plant Material and Extraction Procedure

Aerial parts (A) and flowers (F) of *Rosa banksiae* var. *banksiae* Ait. (RBW) known as Banksian rose were collected from Merryland Botanical Garden, Cairo, Egypt ($30^0$05'37"N$31^0$18'51"E), while *Rosa polyantha* Thunb. orange fairy (RPO) and *Rosa polyantha* Thunb. white fairy (RPW) were collected from a private garden, Al-Mariouteya Road, Giza, Egypt ($30^0$01'13"N$31^0$04'42"E, during March-April 2016 (flowering season) and were authenticated by Eng. Terease Labib, Ministry of Agriculture, Giza, Egypt. Voucher specimens were kept under codes: (PHG-P-RB 165), (PHG-P-RP 205) and (PHG-P-RP 204) for RBW, RPO and RPW, respectively at Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

Aerial parts and flowers (500g for aerial parts and 200g for flowers) of RBW, RPO and RPW were collected, dried in shade then percolated, separately in 2L 70% (v/v) HPLC grade methanol. The filtered extracts were dried *in vacuo* at low temperature (45^0C) till dryness then lyophilized. The lyophilized extracts weighed; 45g, 43.2g and 42.5g for RBW-A, RPO-A and RPW-A, respectively and 20.3g, 19.8g and 15.4g for RBW-F, RPO-F and RPW-F, respectively.

2. Ultra Performance Liquid Chromatography – Electrospray Ionization -Tandem Mass Spectrometry (UPLC-ESI-MS/MS) Analysis

UPLC-ESI-MS/MS in both positive and negative ion acquisition modes were carried out on a XEVO TQD triple quadrupole instrument, Waters Corporation, Milford, MA01757 U.S.A, mass spectrometer. Chromatographic separation of the sample was done by injecting 10µl into UPLC instrument equipped with reverse phase C-18 column (ACQUITY UPLC - BEH, 2.1 × 50 mm column; 1.7 µm particle size). The sample (100 µg/mL) solution was prepared using HPLC grade methanol, filtered using a membrane discfilter (0.2 µm) disc and degassed by sonication before injection then subjected to LC-ESI-MS/MS analysis. Gradient mobile phase comprising two eluents: eluent A is H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid. Elution was made at flow rate 0.2 mL/min as follows: (10%B) from 0 to 5 min.; (30% B) from 5 to 15 min.; (70% B) from 15 to 22 min.; (90% B) from 22 to 25 min. and (100% B) 25-29 min. The analysis was accomplished using negative ion mode as follows: source

temperature 150°C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440°C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h.

2.1. Data Processing

Mass spectra were recorded in Electrospray ionization (ESI) (negative and positive ion modes) (*m/z* 100–1000). They were processed using Masslynx 4.1 software and tentative identification was done by comparing their retention times (R_t), mass spectra and fragmentation patterns with reported data.

2.2. Chemometric Analysis

LC-MS/MS metaolomic profile was subjected to chemometric analysis. Principal component analysis (PCA) acts as the first step in data analysis in order to provide an overview of all observations and samples to identify and evaluate groupings, trends and strong outliers. Hierarchal Cluster Analysis (HCA) was then applied to allow clustering of different *Rosa* species. The clustering pattern was conducted usingcomplete linkage method for group building and computed by Euclidean method. For PCA and HCA, Unscrambler® X 10.4 from CAMO (Computer Aided Modeling, AS, Norway) was applied. A clustered heatmap was constructed using NCSS. 12 software with Euclidean distance and the unweighed pair group method.

3. Determination of Insecticidal Activity

3.1. Larvicidal and Pupicidal Activities

Culex pipiens larvae were collected, during summer 2016, from Sadat City, Cairo-Alexandria desert road and were grown for several generations in Medical Entomology Insectary, Animal House, Department of Zoology, Faculty of Science, Al-Azhar University under standard experimental procedure adopted to provide third instar larvae for the bioassay.

Standard methods [35] for evaluating the larvicidal and pupicidal activity of plant extracts against *Cx. pipiens* larvae were followed with small modifications. *Rosa* extracts were dissolved in 0.1ml methanol to enhance the dissolving in 250ml dechlorinated tap water in 350ml plastic cups. Then, 3rd instar larvae (25 larvae) were placed in plastic cups containing the extracts at different concentrations. Three replicates were used for all tested concentration. All plastic cups were incubated under controlled conditions and subsequently mortality was recorded; control larvae received 0.1ml methanol in 250ml water. Mortality was calculated daily where dead larvae and pupae were removed until the emergence of adults. Larval mortality was confirmed when larvae showed no response to mechanical stimulation and estimated using equation of Briggs[36].

3.2. Reproductive Potential of Resulted Females

Females (3rd instar larvae) treated with tested concentrations of *Rosa* extracts were collected and kept with normal adult males in wooden cages and fed on10% (w/v) sucrose solution for 3 days. They were left for one day without sugar solution then at the 5th day, the starved females were allowed to take a blood meal from a pigeon and allowed to lay egg rafts on clean water (oviposition traps) in the cages. The number of egg/raft was counted using binocular and then mean values were taken. Under a dissecting microscope, the non-hatched embryonated eggs were identified by the apparent confirmation of the embryo presence [37]. Sterility percentages were calculated according to Toppozada formula [38].

3.3. Statistical Analysis

Data were presented as Mean ± SD. Student t-test and one way analysis of variance (ANOVA) was performed using Sigmaplot version 11.0. LC_{50} values were calculated through multiple linear regressions [39].

Conclusion

The metabolomic profiling for the tested species belonging to genus *Rosa* had showed their richness in phenolic secondary metabolites which played an important role in their larvicidal, pupicidal activities and in decreasing the reproductive power of the female *Cx. pipiens*. The identified metabolites were successfully grouped into four clusters through PCA and HCA tools and the individual components were constructed into colored clustered heatmap

depending on the area % values. Finally, the tested *Rosa* species can be regarded as a renewable source for new safe, effective and economic mosquitocidal agent. To further support the profiling method of the selected species, we may carry out isolation of the major components responsible for these biological activities in the future.

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Authors' Contributions

E.A.E. performed the experiments and wrote the article; **N.M.M.** analyzed the data and revised the manuscript, **A.Z.I.S.** performed the biological part**, R.M.L.** analyzed the data and revised the manuscript and **A.B.S.** supervised the whole work and revised the manuscript.

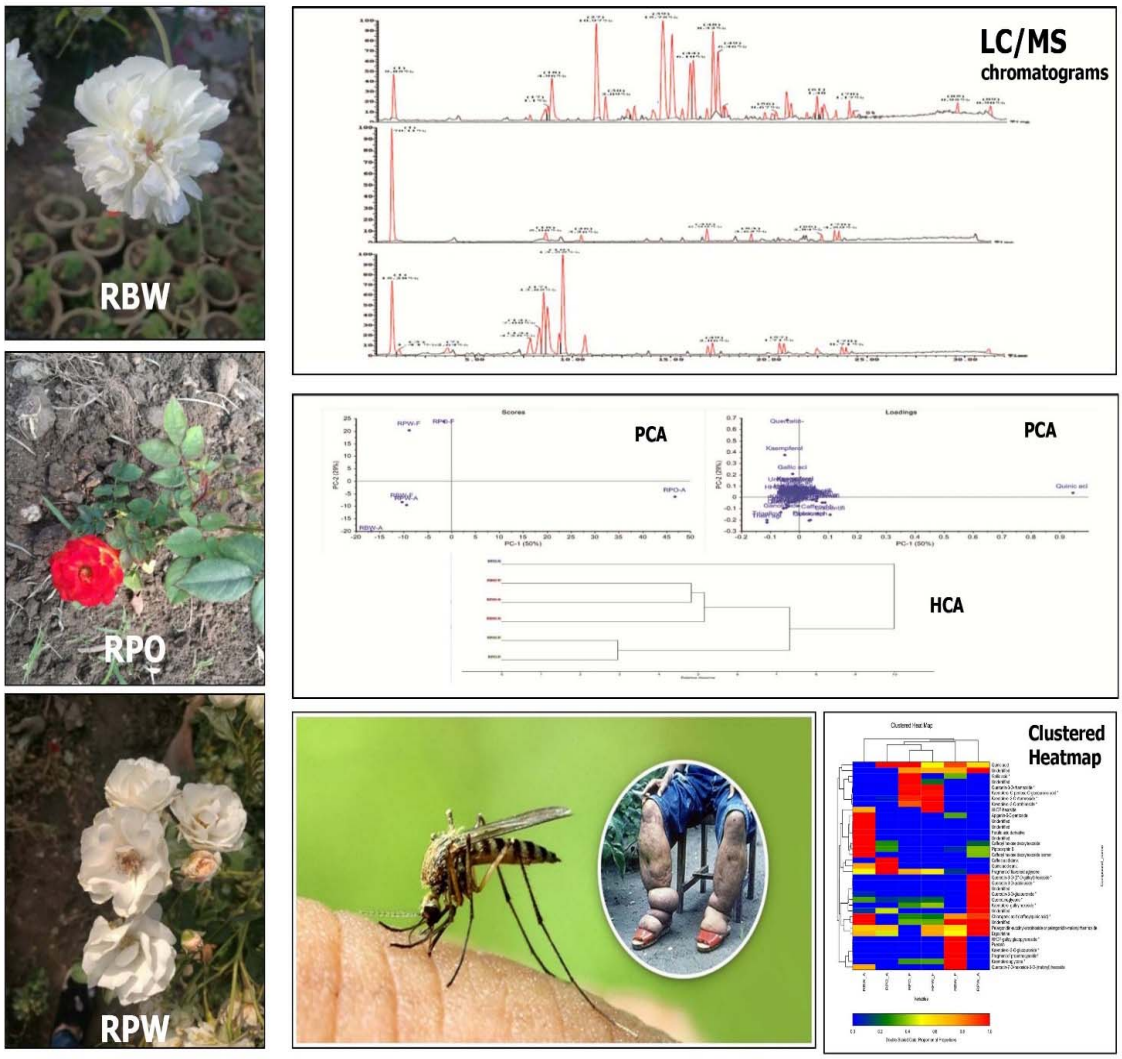
Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of interest

The authors declare no conflict of interest.

Figures



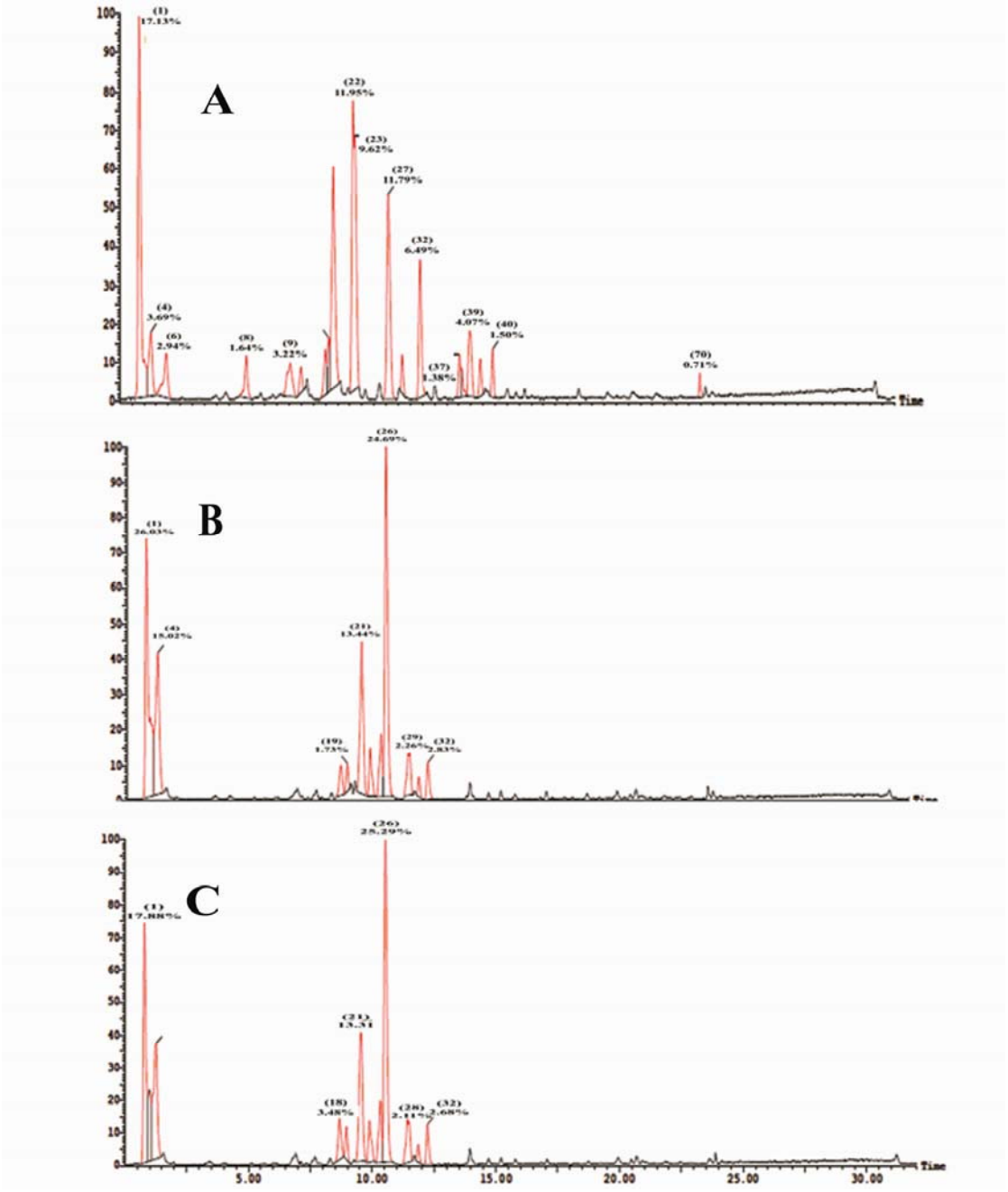


Fig. A. 1 LC/ESI/MS negative ion mode spectrum of (A) *R. banksiae var. banksiae* Ait. aerial parts (RBW-A) methanolic extract; (B) *R. polyantha* Thunb. orange fairy aerial parts (RPO-A) methanolic extract and (C) *R. polyantha* Thunb. white fairy aerial parts (RPW-A) methanolic extract

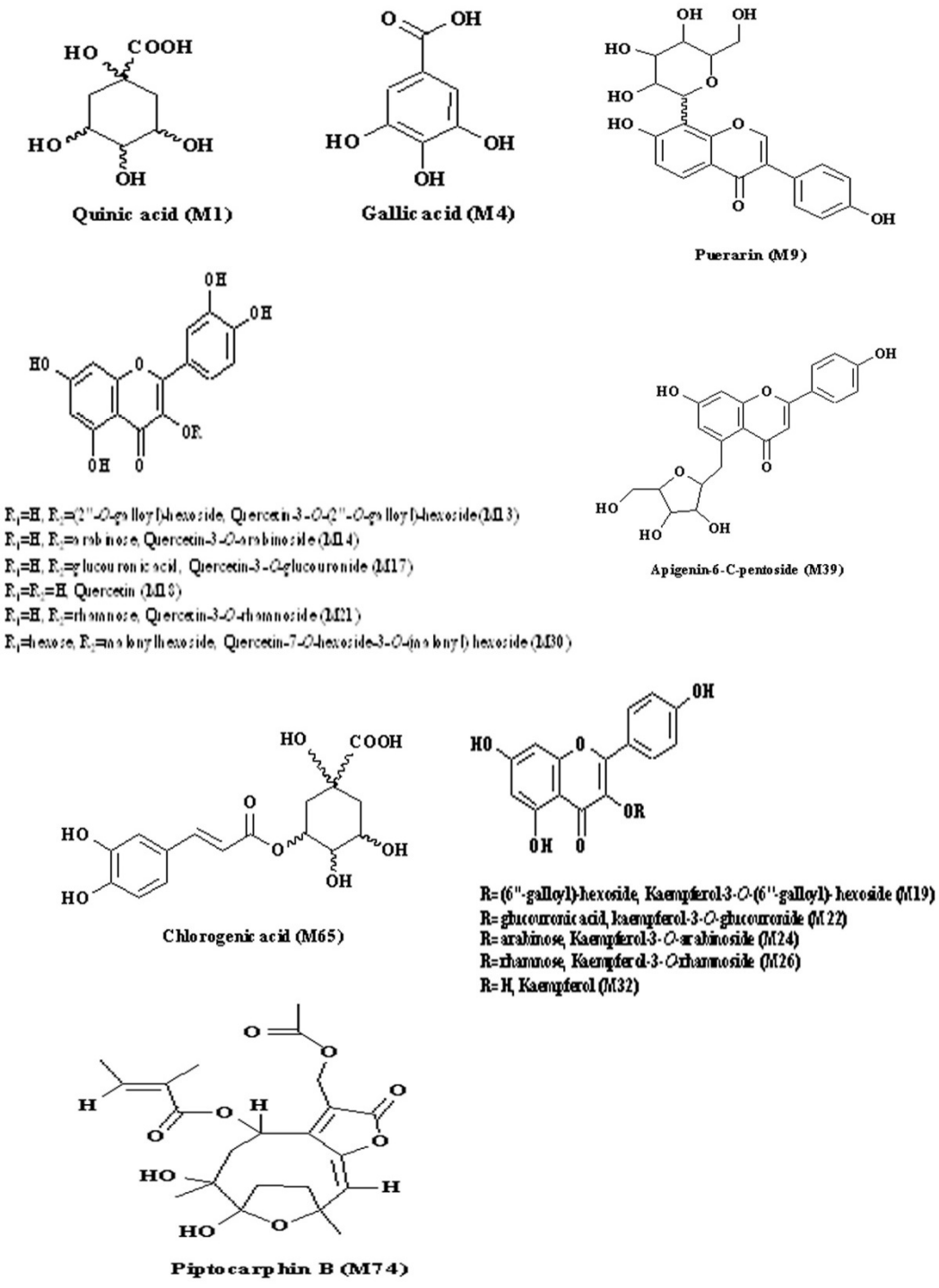


Fig. A. 2 LC/ESI/MS negative ion mode spectrum of (A) *R. banksiae var. banksiae* Ait. flower (RBW-F) methanolic extract, (B) *R. polyantha* Thunb. orange fairy flower (RPO-F) methanolic extract and (C) *R. polyantha* Thunb. white fairy flower (RPW-F) methanolic extract

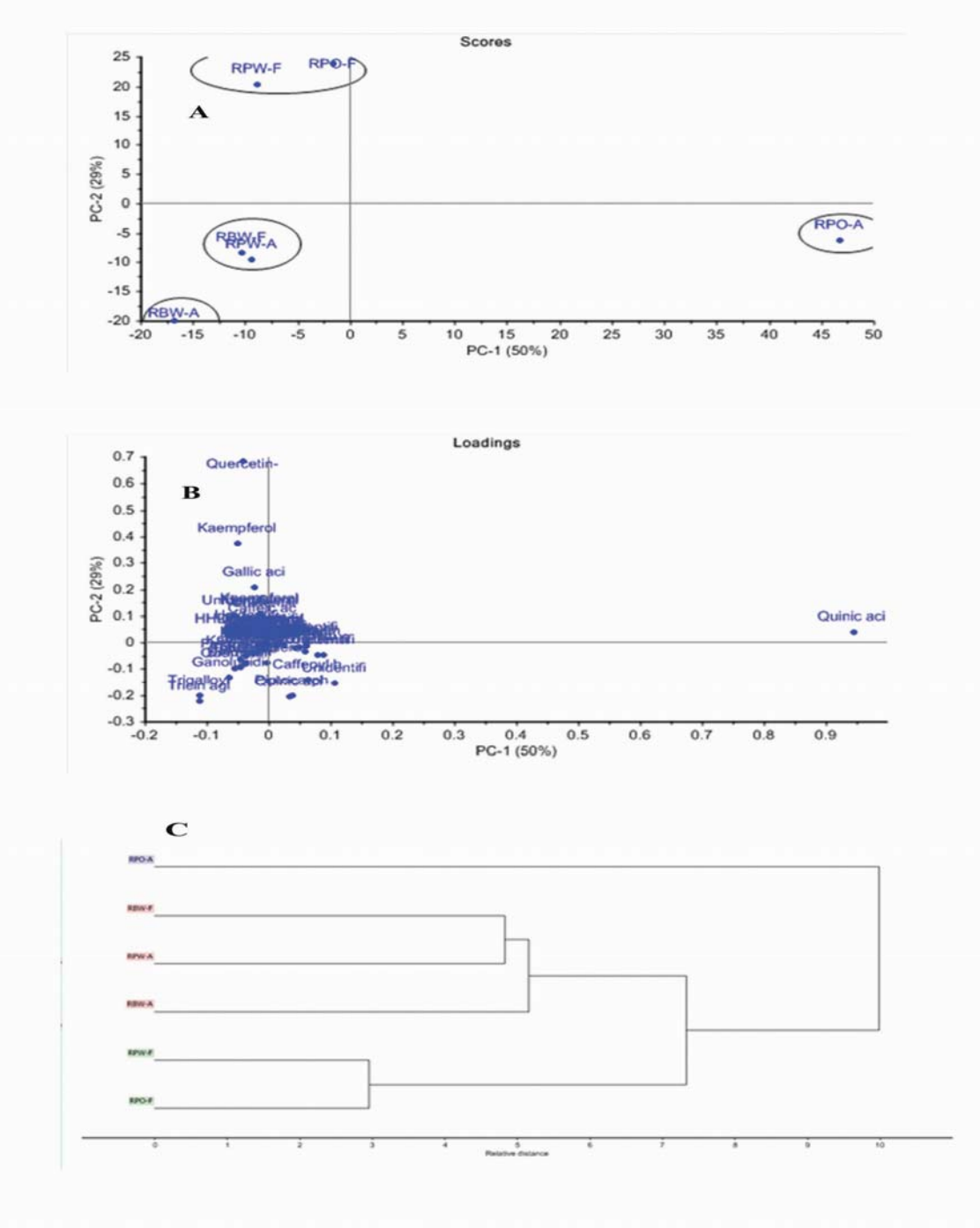


Fig. A. 3 Chemical structures of the major tentatively identified compounds from the 70% methanolic extracts of the tested *Rosa* varieties through LC/ESI/MS^n.

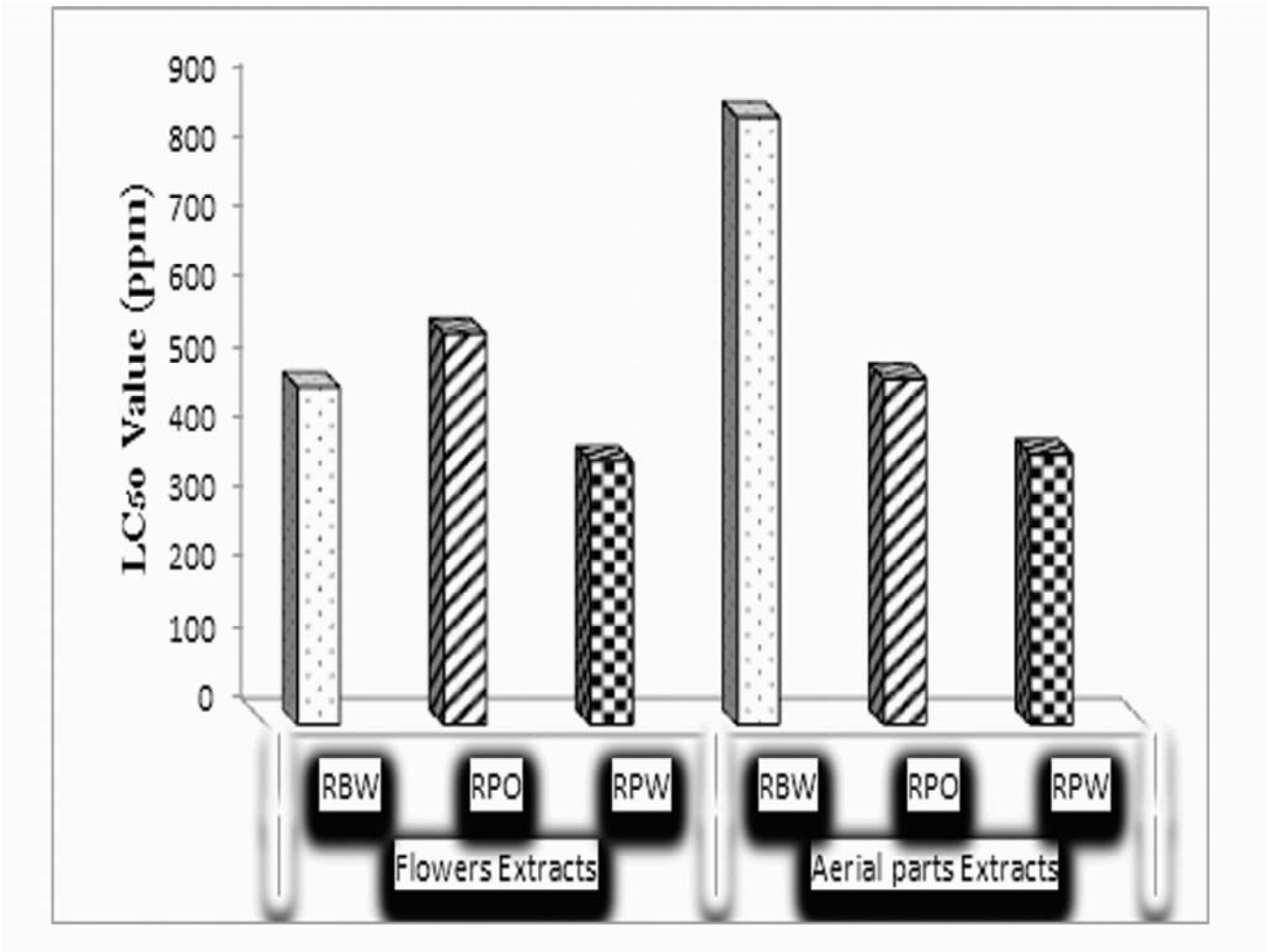


Fig. A. 4 (A, B) PCA score and loading plots of the tested *Rosa* varieties, (C) HCA dendogram of of the tested *Rosa* varieties.

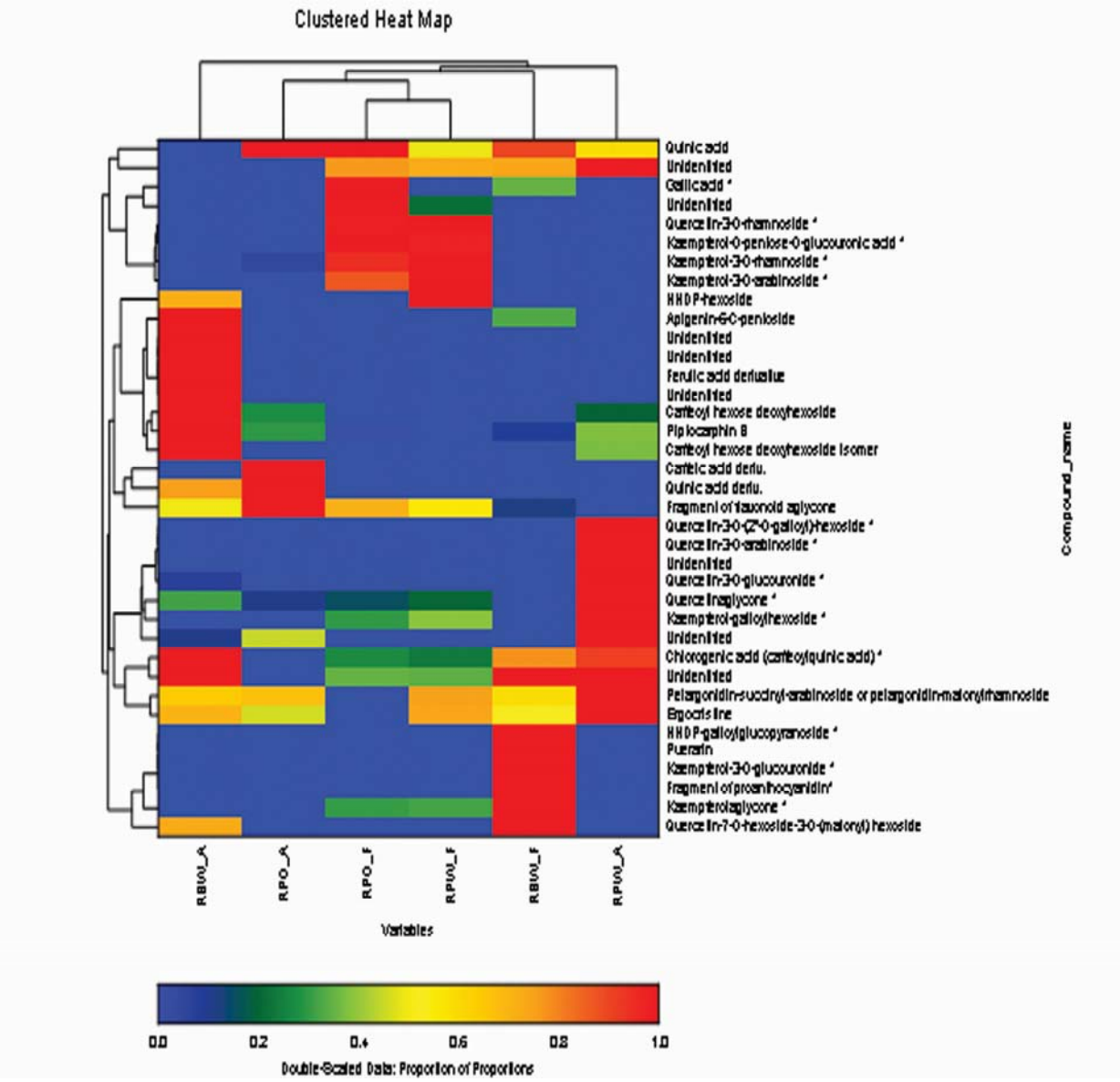


Fig. A. 5 Half Lethal Concentration (LC_{50}) values of methanol extracts of different *Rosa* species against 3rd instar larvae of *C. pipiens* (*R. banksiae var. banksiae* Ait. (RBW), *R. polyantha* Thunb. orange fairy (RPO) and *R. polyantha* Thunb. white fairy (RPW).

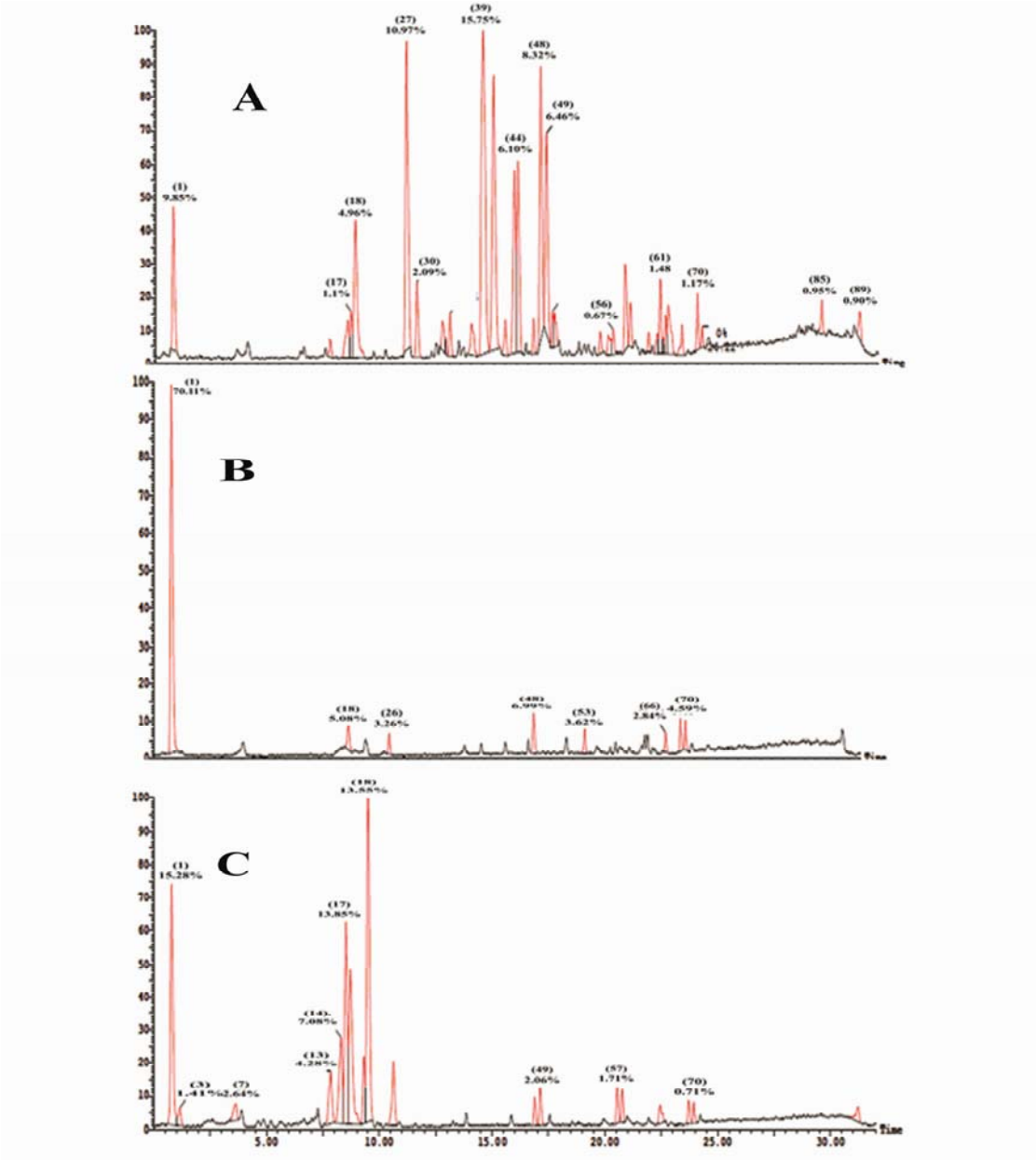


Fig. A. 6 Clustered heatmap showing different metabolites of the six studied *Rosa* samples (Heat map was constructed using Euclidean distance and the unweighed group method. Compounds with % composition of at least 3% were included).

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دراسة مقارنة بين نواتج الأيض لأنواع مختاره من روزا من خلال تحليل الكتلة عالي الكفاءة والكيموميترى وتقييم نشاطهم كمبيدات حشريه ضد كيولكس بيبيينز

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ملخص

باستخدام تحليل الكتلة عالي الكفاءة تم القياس الكمي لمستخلصات الميثانول بتركيز 70 % لثلاث تنوعات من روزا (الأجزاء الهوائية والورود) وهم روزا بانكزيا تنوع بانكزيا، روزا بوليانسا "الورد البرتقالى" وروزا بوليانسا "الورد الأبيض" وتم التعرف وتحديد نسبه 61 مركب أغلبهم من جلايكوسيدات الفلافونويد. تم تقسيم العينات الست إلى أربعه أقسام باستخدام تحليل المكون الأساسي وتحليل المكون الهرمى وهم RBW-F & RPW-A), (RPW-F & RPO-F), RPO-A and RBW-A وقد تم قياس نشاط الست عينات كمبيدات حشريه ضد كيولكس بيبيينز طورا اليرقات والشرانق وكذلك قدرتهم على تقليل الخصوبة للحشرات البالغة. تمكنت مستخلصات الأجزاء الهوائية والورود ل RPW من قتل اليرقات والشرانق بأعلى كفاءه LC50 373.3 and 383.2 ppm لكل منهما تباعا ووصل مؤشر العقم إلى 51.4% عند أعلى تركيز مستخدم كذلك كانت مستخلصات الورود أقوى من مستخلصات الأجزاء الهوائية. ومما سبق يمكن أن نستخدم مستخلصات RBW, RPO and RPW كمبيدات حشرية من أصل نباتي آمنه وفعاله ضد ناقل داء الفيل الكيولكس بيبيينز.

الكلمات الدالة: روزا، تحليل الكتله عالى الكفاءه، نواتج الأيض، كيولكس بيبيينز، داء الفيل .

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Anti-Vibrio from Ethyl Acetate Extract of Sponge- Associated Fungus *Trichoderma longibrachiatum*

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ABSTRACT

Some of *Vibrio* spp. bacteria are pathogenic to humans and other organisms, including cultured fish or shrimp. This study aimed to determine the activity of ethyl acetate extract of *Trichoderma longibrachiatum* as an anti-vibrio fungus. The test bacteria used were: *Vibrio harveyi, Vibrio anguillarum, Vibrio vulvinificus,* and *Vibrio parahaemolyticus*. The fungus was cultured using Malt Extract Agar (MEA) medium for 9 days at 27°C (static conditions, 24 hours dark, pH 5.6, and salinity 60 ppt). Extracts obtained by maceration using ethyl acetate, then the extract is partitioned using water-methanol 70:30 and ethyl acetate. Each fraction was concentrated to obtain polar-ethyl acetate (PE) and semipolar-ethyl acetate (SPE) extracts. The components of the constituent extract were traced with a Thin Layer Chromatography (TLC) and followed by ultraviolet spectrophotometry. The anti-vibrio activity was determined based on the value of Minimum Inhibitory Concentration (MIC). The results of the study showed that SPE was more potential to be used as anti-vibrio. The strongest activity was able to inhibit the growth of *Vibrio vulvinificus* with 256 µg mL^{-1} MIC value, while the weakest was against *Vibrio parahaemolyticus* with 1.024 µg mL^{-1} MIC value. In conclusion, SPE has the potential to be developed as an anti-vibrio compound, particularly against *Vibrio vulvinificus*.

Keywords: Semipolar-ethyl acetate extract, Minimum Inhibitory Concentration, *Vibrio vulvinificus*.

INTRODUCTION

Vibrio is a genus of bacteria found in a variety of freshwater and marine habitats. There are more than 100 species of *Vibrio* spp., and 12 of which cause infection in humans. *Vibrio cholerae* (*V. cholerae*) can cause cholera, a severe diarrheal disease that can be transmitted through contaminated water. Non-cholera *Vibrio* spp., such as *V. parahaemolyticus, V. alginolyticus,* and *V. vulnificus,* may cause vibriosis, an infection with various clinical expressions, and the mildest of which is gastroenteritis. Vibriosis infection is usually caused by exposure to seawater or by consuming contaminated raw or undercooked seafood. There is an exception, *V. vulnificus* which is an opportunistic pathogen causing wound infection which can rapidly lead to septicemia (1, 2). Infections caused by *V. vulnificus* are generally fatal. Therefore, accurate diagnosis and direct treatment are very important since the infection may cause death to the sufferer (3). antibiotics are widely used to treat vibriosis. The commonly used antibiotics include doxycycline, quinolone, cephalosporin, ciprofloxacin, tigecycline, and

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ceftazidime (2, 3). *Vibrio* spp. are gram-negative bacteria that causes vibriosis in humans and animals. Moreover, fishery products and post-harvest products are common intermediaries for the transfer of bacteria from their original habitat to their new hosts (5).

The fisheries sector is one of the most fundamental fields for a country's food security. On the other hand, fisheries systems also play a major role in spreading vibriosis to humans. Vibriosis is a bacterial disease reported in Indonesian marine fish culture since the 1990s. The disease is reported mostly found in grouper (*Epinephelus* sp.) and shrimp (*Penaeus monodon*, *Litopenaeus vannamei*) culture, although infection also occurs in snapper (*Lates calcarifer*) and abalone (*Haliotis squamata*) culture. The agent causing vibriosis in marine fish in Indonesia involves several vibrio species, including *V. harveyi, V. anguillarum, V. alginoluticus, V. parahaemolyticus* (4). Some efforts are done to control vibriosis in fish farming activities still rely on the use of drugs or antibiotics given through oral and immersion.However, feed residues will cause pollution in the waters due to antibiotic contamination. Nowadays, several Vibrio species are even resistant to certain types of antibiotics. Based on the research (5) conducted on the North Coast of Java found that *V. parahaemolyticus* contaminating vanname shrimp is resistant to erythromycin (90%), amoxicillin-clavulanic acid (83.33%), and nitrofurantoin (58.33%).

Vibrio spp. live in marine habitats along with other bacterial species and microorganisms, like fungi. Competition among the inhabitants of an ecosystem will occur to compete for space and nutrition. Consequently, there is a possibility that there are any antagonistic species, both within among themselves and towards other species. Some research resulted in the fact that several marine-derived fungi species synthesize secondary metabolites of anti-vibrio as their chemical weapons to compete and to avoid predation. Several anti-vibrio metabolites found are such as ethyl acetate extract from sponge-associated fungus *Tricoderma asperellum* (6); prenylxanthone and aspergixanthones from the marine-derived fungus *Aspergillus sp*. cultured with shaken Czapek-Dox media (7); indole-diterpenoids and steroids isolated from *Penicillium janthinellum* (8), secondary metabolites of the mangrove-associated fungus *Aspergillus sp*. (9), and secondary metabolites of marine invertebrates associated fungi *A. flavus, A. oryzae, A. aculeatus, Talaromyces minioluteus, Hypocrea jecorina, Gliomastix murorum, Myrothecium inundatum,* and *Curvularia avinis* cultured with Poor Marine Agar (PMA) (10).

The use of antibiotics for a certain period can cause some problem related to pathogenic bacteria resistance towards these antibiotics whether in fish or shrimp body and in addition to the residue which will pollute the environment. Besides, this will also harm humans' health by consuming contaminated sea products. Replacing antibiotics with natural compounds that have antibacterial activity against *Vibrio* spp. offers a new environmentally friendly solution. The potential of sponge-associated fungus *T. longibrachiatum* as a producer of antibacterial compounds has been examined. According to (11), the study results showed that ethyl acetate extract was able to inhibit the growth of several types of both gram-positive and negative bacteria. *Vibrio* is categorized as a member of the gram-negative bacteria group. Accordingly, it is presumed that the extract is also able to inhibit its growth. This study aimed to determine the antibacterial activity of the ethyl acetate extract of sponge-associated fungus *T. longibrachiatum* as anti-vibrio.

MATERIAL AND METHODS

Fungus Isolate

The samples of isolate used in this study were sponge-associated fungi coded TE-PF-03.1. The sponges were collected from the waters of Falajava Beach, Ternate Island, North Maluku, Indonesia (coordinates 00º47'09.12" N; 127º 23'21.76" E) at 3-30 m depth. The fungus has been investigated and identified molecularly using Internal Transcribed Spacer (ITS) rDNA sequence as *T. longibrachiatum* and has been morphologically confirmed (11).

Fungus Culture

Regeneration of fungus collection was carried out before the culture process (i.e. subculture for 7 days). Isolate of *T. longibrachiatum* TE-PF.03.1 was cultured using Malt Extract Agar (MEA Merck) media in 20 Petri dishes. Preparation of MEA was carried out using sterile seawater. According to (11), the final condition of the media showed that the salinity was 60 ppt and pH 5.6. The culture was carried out for 9 days in the environmental condition with 24 hours dark, static, and 27^{o}C temperature.

Secondary Metabolites Extraction

Following 9 day-culture, the media and the micelle were cut into small pieces and macerated with ethyl acetate (1:1v/v). Ethyl acetate extract was obtained after filtration and evaporation processes using a rotary evaporator (40^{o}C) under low pressure. Some part of the ethyl acetate extract was separated for the initial test of its potential as anti-vibrio, while the remaining extract was partitioned using a separatory funnel with water-methanol 70:30 and ethyl acetate (1:1v/v) solvents. Each fraction of polar-ethyl acetate (PE) and semipolar-ethyl acetate (SPE) extract was concentrated and ready to antibacterial test as anti-vibrio (11).

Profiling Secondary Metabolites

The constituents compound of PE and SPE extracts were predicted using the Thin Layer Chromatography (TLC) method (12). In this process, after the extract being developed using a certain mobile phase, it will produce spots on the TLC plate and are identified using Rf (Retention factor) value. The spots on the TLC plate were visualized with ultraviolet (UV) light 365 nm, 2% vanillin-H_2SO_4, 0.25% ninhydrin in acetone, and 1% ferric (III) chloride in methanol (12, 13). Furthermore, the TLC plate was heated at 110^{o}C for 2-3 minutes. Besides, the visualization was done by UV light (λ200-400 nm) which was equipped with an absorption profile using a UV-Vis spectrophotometer.

Antibacterial Activity Test

Vibrio bacteria used in this study were *V. harveyi, V. anguillarum, V. vulvinificus*, and *V. parahaemolyticus*. The subculture of tested bacteria was carried out in Mueller-Hinton Broth (MHB; Oxoid) and incubated for 24 hours at 37^{o}C. The initial antibacterial bioassay was done by determining the inhibition zone diameter using a disc diffusion assay (14). *Vibrio* bacteria were inoculated in Mueller-Hinton Agar (MHA; Oxoid) media with 0.5 McFarland ($1.5x10^{8}$CFU mL^{-1}) initial density using the swab method. Moreover, the extract preparation was started by making a solution with 50 and 25 mg mL^{-1} concentration in DMSO. Then, 10 µL of extract solution was dropped onto the surface of sterile disc paper (Oxoid, 6 mm diameter), and it resulted in 500 and 250 µg $disc^{-1}$ final concentration. The inhibition zone was measured after 24 hours incubation period at 37^{o}C.

Minimum Inhibitory Concentration Test

Minimum Inhibitory Concentration (MIC) test was carried out on fractions PE and SPE of *T. longibrachiatum* ethyl acetate extract. Broth Dilution method was used to determine MIC value referring to the method with resazurin microtiter assay (REMA) (15, 16). The first, 100 µL of the extract solution in DMSO solvent with the highest concentration was filled in the first well in certain rows. Then, the next well was filled with 50 µL of sterile MHB . Moreover, the test material as much as 50 µL was transferred from the first well to the next well to achieve 10 series of dilution (2,048-4 µg mL^{-1}) . Meanwhile, 30 µL of resazurin solution (Sigma-Aldrich, 0.02% in distilled water) was added to each well, then 10 µL of Vibrio bacterial suspension ($1.5 x10^{8}$ CFU mL^{-1}) were added to each well. Chloramphenicol was used as a positive control (with series concentrations from 64-0.125 µg mL^{-1}). Well the 11th filled DMSO as a negative control and the 12th filled MHB as media control. The microplate was incubated at 37^{o}C for 24 hours. After the incubation period, the well as growth control appeared pink. At last, the MIC value was determined based on the lowest concentration that could inhibit the growth of *Vibrio* spp. and appeared blue.

RESULTS AND DISCUSSION

Based on the results of previous studies, *T. longibrachiatum* cultured using MEA media will reach the peak of its secondary metabolites production on day 7. At the following periods, the production decreased, but the antibacterial activity increased until the 9th day of culture. The ethyl acetate extract metabolite has antibacterial activity through the disc diffusion test (500 µg disc^{-1}) against several gram-positive and negative bacteria, while the methanol extract is inactive (11). Similar results were also found by other researchers stating that the ethyl acetate metabolite extract from endophytic fungal isolates mostly showed higher antibacterial activity than the methanol extract (17).

T. longibrachiatum sponge-associated fungus in this study was cultured using MEA for 9 days. Besides, the anti-vibrio potential of its ethyl acetate extract on disc diffusion test (500 and 250 µg disc^{-1}) can be seen in Table 1 and it shows a inhibition zone formed. This ethyl acetate extract was able to inhibit the growth of *V. harveyi, V. anguillarum, V. vulvinificus,* and *V. parahaemolyticus.*

Table 1. Anti-vibrio potential of *T. longibrachiatum* ethyl acetate extract by using disc diffusion method

Tested Bacteria	Concentration of extracts (µg disc^{-1})	
	500	250
V. harveyi	+	-
V. anguillarum	+	+
V. vulvinificus	+	+
V. parahaemolyticus	+	+

Note: += inhibition zone formed, - = no inhibition zone

Further research was done to determine the polarity of the active compounds carried out by partition method using immiscible solvents. Methanol is added water to increase its polarity, so that it can be separated with ethyl acetate. The profile of the compounds in PE and SPE extracts corresponded to the appearance of several spots on the TLC plate as seen in Figure 1. The spots appeared fluorescent blue when being irradiated by UV indicating that the organic compound had double bonds (diene/polyene or conjugated) (18). Compounds that react positively with vanillin reagent would show certain colored spots (varying colors) characterizing the presence of carbonyl functional groups (ketones, aldehydes). These compounds probably come from phenolic, flavonoids, terpenoids, steroids, fatty acids/essential oils, or high molecular weight alcohol groups. Besides, vanillin reagent is sensitive to the steroid class. If the compound reacts positively with the ninhydrin reagent, the compound contains nitrogen (amines, peptides, or alkaloids). Meanwhile, phenolic group compounds will react positively to ferric (III) chloride reagent (12, 19). The results of the study showed that all spots in ethyl acetate extract were not reactive to ninhydrin and ferric (III) chloride reagents; it is accordingly assumed that they are not from nitrogen or phenolic compounds. Perhaps both extract PE and SPE are terpenoids or steroids.

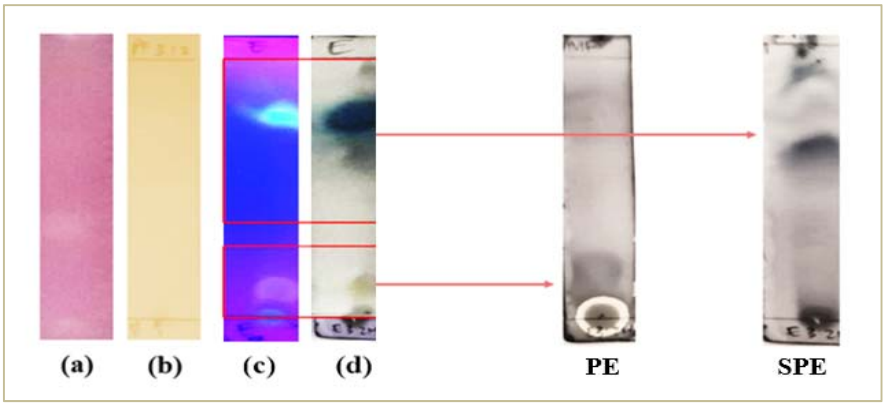

Figure 1. TLC profile of *T. longibrachiatum* ethyl acetate extract and its visualization using: (a) 0.25% ninhydrin, (b) 1% ferric (III) chloride, (c) UV light 365 nm, (d) 2% vanillin-H_2SO_4 (Note: PE=polar-ethyl acetate, SPE=semipolar-ethyl acetate)

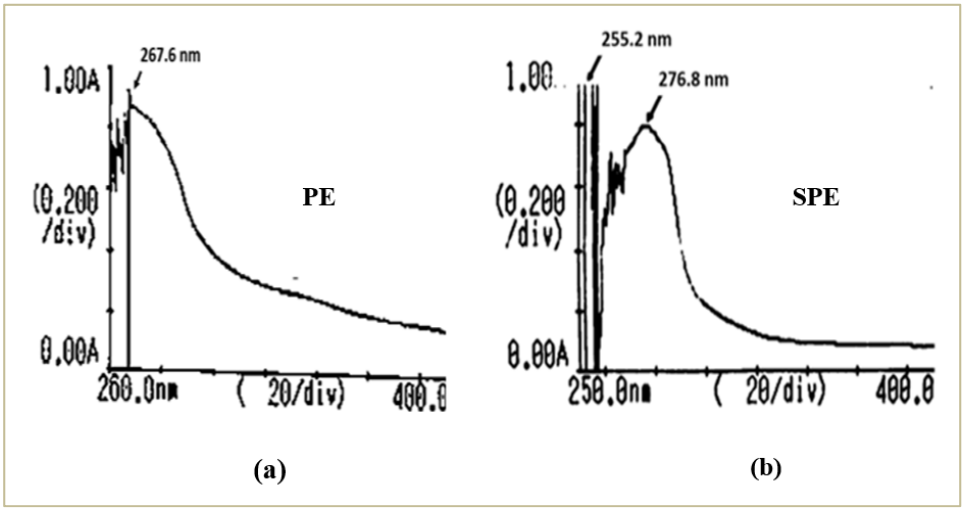

Figure 2. UV spectra (λ200-400 nm) of *T. longibrachiatum* ethyl acetate extract: (a) PE=polar-ethyl acetate , (b) SPE=semipolar-ethyl acetate)

The spectra of UV light absorption is shown in Figure 2. Extract PE has a pattern with 1 peak, i.e. at λ 267.6 (A=0.93), while SPE has 2 clear peaks, at λ 255.2 nm (A=4.00) and λ 276.8 (A=0.80). There may be other peaks in SPE. This was also reflected in the TLC profile of SPE which was observed in more than 2 spots after visualization with 2% vanillin-H_2SO_4 (as in Figure 1d). Based on the literature study, some terpenoids and steroids possess a carbonyl functional group and also conjugated double bonds. This assumption is strengthened by observing the UV spectra patterns of each PE and SPE. The result of previous research (20), the UV spectra of terpenoids seems to have an absorption peak at λ 259.57 nm for monoterpenoids (C10) compounds which are assumed to be thymol and at λ 260.47 nm for sesquiterpenoids (C15) which is assumed to be chiloscyphone. The structure of chiloscyphone compounds contains ketone functional groups and conjugated double bonds, whereas thymol only has conjugated bonds. This assumption was strengthened by previous statements (21), that

conjugated double bond which also has a carbonyl functional group will produce chromophores that intensively absorb UV light in λ 230-270 nm range, and weakly at 300-330 nm. Meanwhile, regarding the chemical structure of steroids, 2 double bonds can be distributed between two adjoining rings (heteroannular diene). In this case, the steroid will absorb in the UV region at 220-250 nm. Moreover, it is also possible that 2 ethylene bonds ($H_2C = CH_2$) are in the same ring (homoannular dienes). Consequently, this will shift the absorption peak to 260-285 nm.

Based on the data in the following Table 2, the SPE obtained from *T. longibrachiatum* has better anti-vibrio activity than the PE. It has strongest potential against *V. vulvinificus* with a 256 μg mL^{-1} MIC value. There are only 2 species sensitive to chloramphenicol, i.e. *V. harveyi* and *V. vulvinificus*, and 2 others that are categorized in the intermediate group. The anti-vibrio potential of SPE is still weak; this is presumably because the extract is not yet pure. The extract is still in the form of a mixture of several compounds which may not be fully synergistic in supporting its antibacterial properties.

Table 2. MIC values of polar-ethyl acetate (PE) and semipolar-ethyl acetate (SPE) extracts against *Vibrio* spp.

Tested Bacteria	MIC value against *Vibrio* spp. (μg mL^{-1})		
	PE	SPE	Chloramphenicol*
V. harveyi	> 2,048	512	8
V. anguillarum	> 2,048	512	16
V. vulvinificus	1,024	256	2
V. parahaemolyticus	1,024	1,024	16

Note: * Categories on organism as susceptible (22):
MIC>32 = resistant, 16-32 =intermediate, <8 =sensitive

Based on various results of other previous studies, there are many terpenoids isolated from fungi. *Sesquiterpenes, meroterpenes*, and *diterpenes* make up the largest proportion of *terpenes*. The genera of *Penicillium, Aspergillus*, and *Trichoderma* fungi are terpenoid's dominant producers. The majority of fungi isolated from living material from the sea (animals and plants) produce terpenoids and many exhibit various bioactivities, such as cytotoxicity, anti-inflammatory, enzyme inhibitors, including antibacterial activities (23, 24). Similarly in butanolic extract of terrestrial *Trichoderma sp*. (isolated from forest plants), it is mostly dominated by terpenoid compounds identified as terpenes (limonene) (92.6%). Other constituents represent a small amount proportion consisting of hydrocarbons (2.01%), alcohol (2.4%), ketones (1.78%), and esters (1.03%). Culture activity using Potato Dextrose Agar (PDA) media produces more terpenoid metabolites than MEA. The growth of *Staphylococcus aureus, Staphylococcus epidermidis,* and *Micrococcus luteus* was inhibited at 500 μg mL^{-1} concentration, while the growth of *Escherichia coli* was inhibited at 1 mg L^{-1} concentration (25).

The compounds in extract SPE were relatively less polar; as the character of terpenoids or steroids which are composed of isoprene (C5) hydrocarbon and were presumed to have a carbonyl or hydroxyl group. The presumption of antimicrobial properties is according to (26) stating that oxygenated terpenes show strong antibacterial activity, particularly against gram-negative bacteria. The increase in antimicrobial activity is associated with the presence of hydroxyl functional groups (phenolic compounds or alcohols), while the hydrocarbon groups produce relatively low activity. The bacteria which could be inhibited were *S. aureus, Bacillus cereus, E. coli,* and *Salmonella enterica*.

Some oxygenated terpene compounds such as carvacrol, l-carveol, eugenol, trans-geraniol, and thymol showed higher activity when compared to sulfanilamide. Terpineol showed excellent bactericidal activity against *S. aureus* strains. Meanwhile, carveol, citronellol, and geraniol exerted a rapid bactericidal effect against *E. coli*. The images obtained by scanning electron microscopy (SEM) show that the mechanism which causes the death of the bacterial cell is based on the loss of integrity on cellular membrane function.

The steroid group and its derivatives isolated from several strains of fungi have also been examined and the results of the study showed antimicrobial activities. Steroids are also produced by *Trichoderma sp., Penicillium sp.,* and *Acremonium sp.* with their greatest abundance respectively are ergosterol, ergostatetraenol, ergostapentaene, neoergosterol, and eburicol. Ergosterol is a sterol (alcohol steroid) commonly found in the plasma membrane of fungi. These steroids have antimicrobial activity against several gram-positive and negative bacteria, for instance. *S. aureus, Bacillus sp., B. cereus, Listeria ivanovii, E. coli, Citrobacter freundii*, and *Salmonella* spp. However, their antimicrobial activity is weak against all bacteria with highest inhibition zone for eburicol (24 mm) (27). Similar results were also seen in this study, extract SPE was able to inhibit the growth of *V. harveyi, V. anguillarum, V. vulvinificus*, and *V. parahaemolyticus*, but with high MIC values, ranging from 256-1,024 μg mL^{-1}.

CONCLUSION

Semipolar-ethyl acetate (SPE) extract from sponge-associated fungus *T. longibrachiatum* has stronger antibacterial activity than its polar- ethyl acetate (PE), so it is concluded that SPE has the potential to be developed as an anti-vibrio compound. Besides, the strongest potential of SPE was able to inhibit the growth of *V. vulvinificus* with 256 μg mL^{-1} MIC value, while the weakest was against *V. parahaemolyticus* with 1,024 μg mL^{-1} MIC value.

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المضادة للفيبريو من استخراج خلات إيثيل من جمعية الفطر–الإسفنج *Trichoderma longibrachiatum*

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ملخص

بعض أنواع البكتيريا .*Vibrio* spp هى مسببات الأمراض للبشر والكائنات الحية الأخرى، بما فى ذلك الأسماك المستزرعة أو الروبيان. هدفت هذه الدراسة إلى تحديد فعالية مستخلص أسيتات الإيثيل من *Trichoderma longibrachiatum* مثلالمضادة لVibrio spp. كانت بكتيريا الاختبار المستخدمة هى: *Vibrio harveyi* , *Vibrio anguillarum* , *Vibrio vulvinificus* , *Vibrio parahaemolyticus*. تمت زراعة الفطر باستخدام وسط مستخلص الشعير لمدة 9 أيام عند 27 درجة مئوية (ظروف ثابتة، 24 ساعة مظلمة، درجة الحموضة 5.6، و الملوحة 60 لكل ألف). يتم الاستخراج الأولي عن طريق التكسير باستخدام خلات الإيثيل، ثم تتركز. يتم تقسيم المقتطف مع المذيبات الميثانول أكواديس (70:30) وخلات الإيثيل بنسبة 1:1. القطبية الإيثيل. ويتركز كل جزء بحيث يتم الحصول على مقتطفات خلات الإيثيل القطبي ومستخلصات خلات شبه القطبية الإيثيل. يتم تتبع مكونات المركبات المكونة لها باستخدام طريقة الكروماتوغرافيا طبقة رقيقة. نشاطالمضادة لVibrio يتم تحديده على أساس الحد الأدنى لقيمة التركيز المثبطة باستخدام اختبار ريسازورين. وأظهرت النتائج أن مقتطفات خلات شبه القطبية الإيثيلية هي أكثر قدرة على مكافحة Vibrio. أقوى نشاط لها قادر على منع نمو V. vulvinificus مع الحد الأدنى من قيمة تركيز المثبطة من 256 جزء في المليون، في حين أن أضعف ضد V. parahaemolyticus بقيمة 1024 جزء في المليون. المركبات التي يعتقد أنها بمثابة المضادة للفيبريو هي تيربينويدات أو المنشطات. في الختام، يمكن تطوير خلات استخراج الإيثيل شبه القطبي كمركب مضاد للفيبريو، وخاصة ضد V. vulvinificus.

الكلمات الدالة: استخراج خلات شبه القطبية الإيثيلية، الحد الأدنى من التركيز المثبط، *Vibrio vulvinificus*.

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The Role of Pharmacists in Patient Counselling for OTC Medication in Jordan: A Cross-Section Study

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ABSTRACT

Background: Community pharmacists represent the easiest-to-access medical experts for drugs. They play a major part in educating and counselling patients, especially regarding over the counter (OTC) medications.
Purpose: This study aims to explore the role of pharmacists in patient counselling for OTC medication in Aqaba, Jordan.
Methods: A cross-sectional survey was conducted with community pharmacists in Aqaba. An online self-administered survey was launched for the study sample via a social media platform (i.e. WhatsApp). The responses were imported into the Statistical Package for the Social Sciences (SPSS) for statistical analysis.
Results: About half of pharmacists started their counselling by asking about the patient's history. More than 70% of pharmacists advised patients in terms of their dosage regimen, the proper indications for the OTC medication, and any possible food-drug interactions. However, approximately one-third of pharmacists suggested there were many challenges in the counselling process. These included limitations in counselling time, work overload, more patients than the pharmacist's capacity and a lack of counselling area.
Conclusion: This study illustrates that community pharmacists are highly committed to pursuing their pharmaceutical care role through proper counselling for OTC medication in Aqaba. Moreover, our study highlights some challenges that pharmacists could face, which may interfere with the efficacy and safety of the drugs they provide.
Keywords: Community pharmacy, Counselling, Jordan, OTC medications.

INTRODUCTION

Worldwide, pharmacists are part of the healthcare workforce and have a trusted role, in which patients are counselled directly, with the pharmacist providing any advice and information they need [1]. Thus, the pharmacist is a key professional in provision of patient education and counselling, as the health professional who is easiest to access [2]. The American Society of Health-System Pharmacists (1997) defines counselling patients as "providing information orally or written form to the patient or his/her representative on direction of use, advice on side effect, precaution, storage, diet, and lifestyle modification" [3]. Appropriate counselling from pharmacists has resulted in patients adhering to their treatment more closely, helped to prevent health issues, improved patient satisfaction, and enhanced outcomes clinically and in terms of life quality, as well as improving people's general understanding of medicines and diseases [4].

Within the Jordanian healthcare system, the community pharmacy is the primary care facility which is simplest for patients to access. However, the role of

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community pharmacists is mostly to dispense drugs, and these professionals tend to interact with patients only in a limited way [6]. While the legal framework regarding the dispensing of drugs in this setting is closely related to Western frameworks, in practice, regulations are not subject to strict enforcement [7]. A typical patient visits the community pharmacy in order to buy products, and consults a pharmacist as a cheaper way to diagnose or treat their conditions than visiting a physician's clinic [8].

Recently, patients have tended increasingly to self-medicate using over the counter (OTC) drugs which are available without a prescription from a pharmacy [9]. Moreover, deregulation of increasing numbers of medications has contributed to this trend [10]. The American Pharmacists Association estimates that OTC treatments are used to treat two billion health conditions annually, out of a total of 3.5 billion health problems treated yearly, an estimated 2 billion health problems are treated with the use of OTC products [11]. The World Health Organization (WHO) has defined non-prescription medicines as medicines which the health regulators have authorised for use in treating non-serious symptoms and conditions. OTC drugs can be accessed without being prescribed, based on evidence that they are effective and safe when patients take them correctly as described on their label or accompanying information leaflet [12].

However, these medications are not a without risk, which places pharmacists and their staff in a position of greater responsibility to conduct their role in pharmaceutical care properly [8]. These duties include providing essential information to the patient to allow appropriate selection of medications and encouraging patients to read all relevant information on the labelling of these drugs, as well as asking for help where they are unsure if a product is appropriate, or how to use it [13.14]. Aqaba is in the southernmost area of Jordan, and it is a non-capital coastal city with a population of 203,200, as per the estimated Population at End-year 2018 [15, 16]. In Aqaba, there are 75 community pharmacies, am d among these there are 4 chains, with approximately 16 branches, while the others are independently run.

Aim of the study

The main aim of this study was to examine the knowledge of pharmacists regarding OTC medications, and reports the differences in patient counselling, with a view to provide the best ways to improve pharmacies. As far as the authors could ascertain, this project is the first conducted in Aqaba to explore pharmacists' role in counselling patients for OTC medication in Aqaba, Jordan.

Ethics approval

Ethical approval to carry out the research was given by Al-Balqa' Applied University in June 2019. Patient information was confidential.

Methods

Design and Context

This study uses a cross-sectional and descriptive study design, where data were collected in a self-completed questionnaire accessed through Survey Google Drive. The survey was carried out in Aqaba, Jordan.

Data collection

A cross-sectional online survey was distributed to every pharmacist who was registered with the Jordan Pharmacists Association (JPA) – Aqaba and to the assistant pharmacists within the community pharmacies via 'WhatsApp' massages.

The survey used a self-administered questionnaire written in Arabic, as Jordan's official language, and was carried out from June -August 2019.

Study tool

The questionnaire consisted of 43 questions over 4 parts. In part 1, the following socioeconomic variables were included: gender, age, duration of experience in community pharmacy, educational level and they type of pharmacy they worked in. The second section dealt with the pharmacists' perception of basic OTC-pharmaceutical care aspects, including history of administration and counselling (implication and challenges). Here, respondents had a choice of responses as "never", "rarely",

"sometimes", "often" or "always". The third section was designed to measure the score of pharmacists' perceptions of the importance of certain counselling points (1-5, least - most important). To establish the score of the pharmacists' perceptions of the importance of some of the counselling points, points were allotted for the responses, in which 1 was "least important" and 5 "most important". The last section was an open-ended question to pharmacists to give any suggestions for other pharmacists when providing advice about drugs without a prescription.

The collection of data using these data collection forms was validated by conducting a pilot study in four pharmacies. The data collection form was reviewed by two PhD holders in clinical and pharmacy practice to ensure validity.

Data analysis

Coding of the data took place for all completed surveys, entering this data into the Statistical Package for the Social Sciences (SPSS, version 24, Chicago, IL, USA) software, which was used for statistical analysis. Descriptive statistics, including percentages and frequency distribution, were calculated for each of the questions. Scores were calculated using Microsoft Excel.

Results

A total of 125 pharmacists (corresponding to 63% of the total 180 registered community pharmacies within Aqaba) and 46 assistance pharmacists in Aqaba participated voluntarily in the online survey.

Sociodemographic characteristics

Out of the total completed questionnaires (n= 125), the majority of the participants were females (n=90, 72.0%), and had a wide age-range, with the highest percentage (n=83, 66.4%) aged 20-30 years. Two thirds of the participants (n=55, 44.0%) had 1 to 5 years of experience and n=59, 47.2% had a BSc in pharmacy, followed by a diploma in pharmacy (n=46, 36.8%). Half of the participants worked in chain community pharmacies (n=64, 51.2%) (Table 1).

Pharmacist perceptions of basic OTC-pharmaceutical care aspects

It was found that pharmacists "always" ask about age (n=71 (56.8%), pregnancy or/and breastfeeding (n=79, 63.2%), the purpose of the medication (n=61, 48.8%), and allergy (n=50, 40.0%) while dispensing OTC medication. One-third of pharmacists "often" asked about family history of known diseases (n=39, 31.2%), the patient's current use of certain prescribed medication (n=51, 40.8%), current use of certain OTC medication (n=43, 34.4%), and history of any experienced side effects related to the medication (n=42, 33.6%) (Table 2).

More than half of the study participants always counsel patients about their dosage regimen (n=110, 88.0%), the duration of medication use (n=65, 52.0%), the proper administration method for the nebulizer, suppositories, etc. (n=73, 58.45%), the proper indications of the OTC medication, such as antipyretic, vitamin, etc. (n=93, 74.4%), the effects of administration regarding meals after eating or before eating (n=94, 75.2%).

When asked about the challenges that can hinder the pharmaceutical counselling process, the following was reported. About one-third of the pharmacists confirmed "often" for limitations in counselling time (n=42, 33.6%), shortage in drug-related knowledge (n=27, 21.6%), shortage in disease-related knowledge (n=29, 23.2%), work overload, number of patients exceeding the pharmacy's capacity (n=41, 32.8%), and a lack of counselling area (n=30, 24.0%).

The average score of pharmacist perception of the importance of some counselling was 3.84±2.55 out of 5 (points (1-5, least -most important)), which included "*the explanation of the proper storage conditions of the medication, the proper duration of treatment and use of the medication, contraindications of the medication, the proper administration method of the medication, and the common side effects of the medication and how to deal with it"* (Table 3).

Writing the instructions on the package (83.2%), followed by a conversation (76.0%), pasting ready-made

labels on the package (51.2%), and asking the patient to read the method from the leaflet (13.6%) are the most common counselling methods used among the pharmacists (Figure 1). In addition, the majority of pharmacists reported that self-learning by using the internet and published articles (84.0%), attending specialized lectures (82.4%), attending training courses (70.4%), and attending specialized conferences (57.6%) are the sources of information that they preferred in order to enrich and update their knowledge about OTC medications (Figure 2).

Discussion

Currently, there is an increasing tendency towards self-medication with OTC drugs in Jordan. The need to wait before being examined by physicians, fees for physician appointments and having a condition which is not sufficiently serious are the most common reasons behind self-treatment in Jordan [7, 17]. Accordingly, pharmacists running community pharmacies in Jordan play an essential role in providing patients with adequate counselling regarding correct and safe self-treatment using OTC products. As far as can be ascertained, this study is unique in Jordan in evaluating pharmaceutical counselling of OTC drugs at community pharmacies from the point of view of pharmacists. This study shows that about half of pharmacists ask about age, pregnancy or/and breastfeeding and the purpose of the medication. This helps in the prescription of the correct OTC medication.

Ensuring that recommended patient counselling practices are followed is challenging, involving altering practice at pharmacies and the need to effectively audit and train pharmacists [3]. Therefore, in order for pharmacists to give suitable counselling to patients when dispensing OTC drugs and to enhance service quality at community pharmacies, pharmacists need to have information available to them regarding medications, and need to participate in continued learning [18].

Our study results have revealed that the majority of pharmacists always counsel patients about their dosage regimen (>80.0%), provide drug administration instruction with regards to meals (75.2%), and give information about the proper indications of OTC medications (74.4%). These results indicate an overall good response of pharmacists, since many previous studies have concluded that pharmacists and doctors are patients' main providers of information about over the counter drugs [19]. However, based on the conducted survey only about half of the pharmacists always asked patients about their age, the purpose and duration of taking the medication and only 58.45% offered advice on the proper administration method of the nebulizer, suppositories, etc. Moreover, less than one-third of the pharmacists “often” asked about family history of known diseases, and 33.6% asked about the history of any side effects related to the medication, while unfortunately, one-third of pharmacists report “rarely” asking patients about family history for a known disease. Accordingly, the aforementioned numbers reflect suboptimal counselling among pharmacists regarding the safety of prescribing OTC medication, which is arguably putting patients at high risk of serious medication side effects, contraindications, or allergic drug reactions.

A lack of proper patient counselling in Jordan has been confirmed by other studies. Hammad et al. (2018) conducted a patient simulated study to evaluate the management of headaches by OTC analgesics in 50 community pharmacies, distributed in Amman, Zarqa and Al-Salt. They found that for most visits, the pharmacists did not question patients about their medical history. In another study performed at a Jordanian teaching hospital, the researcher observed a shortage of proper patient counselling regarding the safety of drug prescription in most cases [21].

Barriers to proper pharmaceutical counselling processes reported in our study are that there are “often” limitations in counselling time (33.6%), work overload beyond the pharmacist capacity (32.8%), lack of counselling area (24.0%), a shortage in disease-related knowledge (23.2%) and a shortage in drug-related

knowledge (21.6%). Likewise, other studies [22, 23] in Jordan reported the same counselling barriers with some variations in the frequency of reported problems.

Although the majority of participants reported that they conduct their self-learning using the internet and published articles to enrich their counselling information, a substantial number of pharmacists reported a shortage of knowledge as a limitation. Therefore, we suggest increasing the number of pharmacists per shift as a solution to minimize working overload, thereby, each pharmacist will have more time for patient education and counselling. More importantly, we believe that a comprehensive training program during the undergraduate period focusing mainly on pharmacology and pharmacotherapy should be implemented and students should pass a strict training exam provided by their educational institution to ensure pharmacy students have a sufficient scientific background.

This study has resulted in a number of suggestions to make the counselling of OTC medications better. The respondents highlighted the importance of learning more than one language, as Aqaba is a city based on tourism. Moreover, some pharmacists suggest making OTC counselling a paid service.

The present study has several limitations. It was not possible to determine the response rate because of the electronic distribution method. In addition to this, more than 85% of the pharmacists who took part were ≤40. This may be due to a lower interest level among the older age group (>40 years), or potentially due to lower engagement in social media platforms. The fact that the majority of respondents were women (>70%) may be due to a lower level of interest amongst males in participating in the study. However, it should be noted that the majority of Jordanian pharmacists are women, and until February 2019, women accounted for 64.4% of pharmacists registered in the country, as reported by the pharmacists' association [24].

Conclusion

Counselling regarding OTC drugs is an everyday task for Jordanian pharmacists in Aqaba. In general, our study shows that most pharmacists counsel patients properly in OTC medications. Moreover, this research uncovers an issue in the low-quality counselling from some pharmacists about side effects that the patient could experience after using the medication and how they can deal with them may interfere with the efficacy and safety of drugs.

The long-standing duties of the pharmacist, including tasks in relation to medications and dispensing prescriptions, need to be improved and extended so that the pharmacists can provide their best expertise and knowledge to help patients as much as they can. Thus, there is a need for a broad framework specifying appropriate actions to be taken to improve knowledge levels among pharmacists and equip them to perform effectively across different conditions [25]. It is suggested here that team working, communications channels and training courses should be considered an effective tool to improve and keep pharmacists up to date with new drug developments.

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Funding Statement

No external funding was needed to conduct and complete the study.

Conflicts of interest

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

Figures

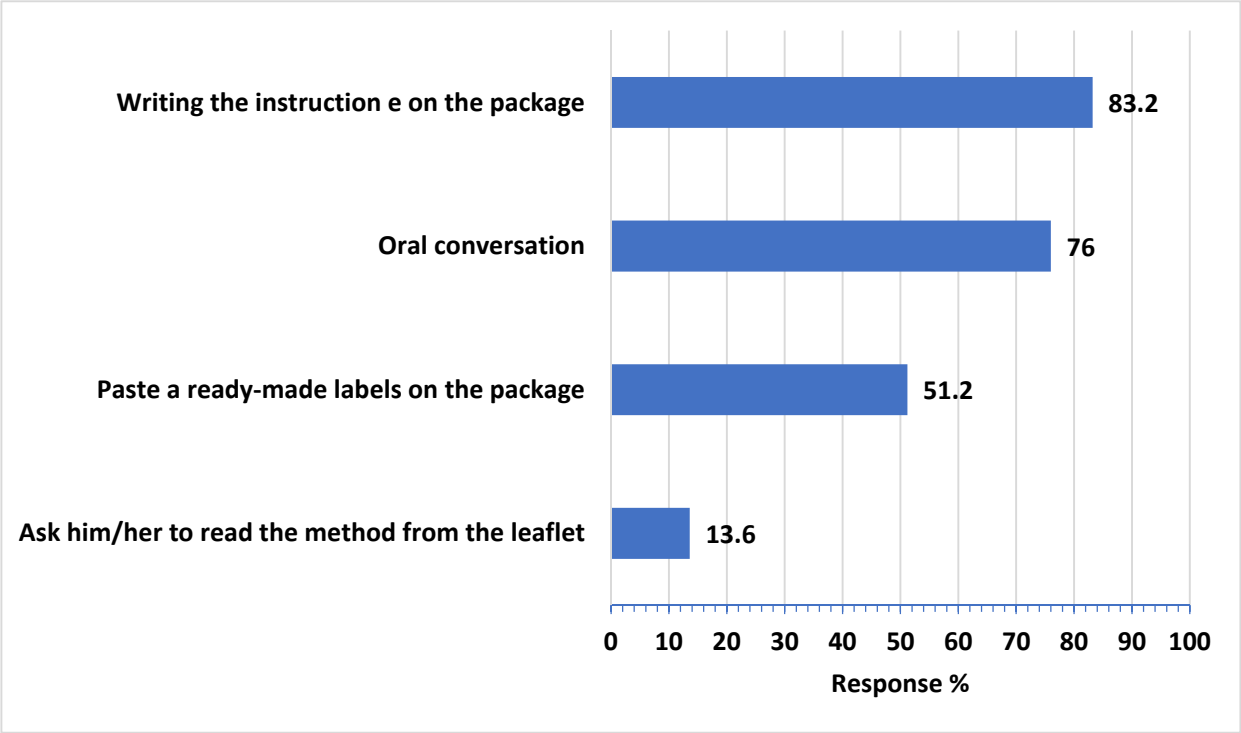

Figure 1. The percentage of the most common counselling methods used among the pharmacist.

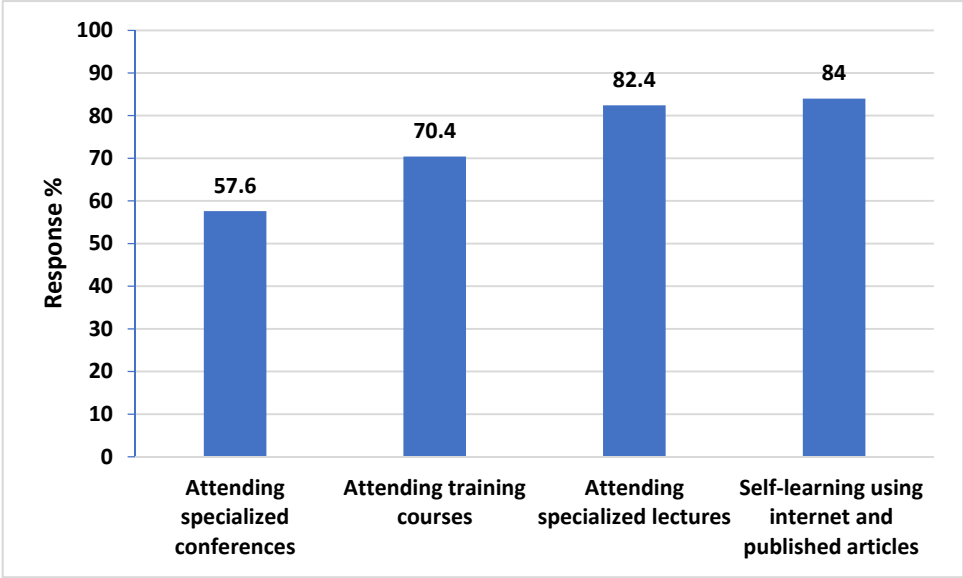

Figure 2. Sources of information that pharmacist preferred to use to enrich his scientific background.

Tables

Table 1. Sociodemographic data for the study participants (n=125)

Variable	n	%
Gender		
· Female	90	72
· Male	35	28
Age		
· 20-30 years	83	66.4
· 31-40 years	25	20
· 41-50 years	13	10.4
· 51-60 years	3	2.4
· More than 60 years	1	0.8
Duration of experience in Community pharmacy		
· Less than one year	24	19.2
· 1 to 5 years	55	44
· 6 to 10 years	18	14.4
· 11 to 15 years	12	9.6
· More than 16 years	16	12.8
Educational level		
· Diploma in pharmacy	46	36.8
· BSc in pharmacy	59	47.2
· Pharm D	13	10.4
· MSc in pharmacy	6	4.8
· PhD in pharmacy	1	0.8
Type of pharmacy working in		
· Individual community pharmacy	61	48.8
· Chain community pharmacy	64	51.2

Table 2. Pharmacist perception for basic OTC-pharmaceutical care aspects including history administration and counselling (implication and challenges)

	n (%)				
Question	**Always**	**Often**	**Sometimes**	**Rarely**	**Never**
Upon dispensing OTC medication, do you ask the patient about it?					
· Age	71 (56.8)	40 (32.0)	14 (11.2)	0.0	0.0
· History of chronic diseases	61 (48.8)	33 (26.4)	27 (21.6)	4 (3.2)	0.0
· The purpose of purchasing the medication	58 (46.4)	49 (39.2)	15 (12.0)	3 (2.4)	0.0
· Pregnancy or breast feeding	79 (63.2)	32 (25.6)	13 (10.4)	1 (0.8)	0.0
· Any allergy to certain foods or medicines	50 (40.0)	34 (27.2)	28 (22.4)	13 (10.4)	0.0
· Current Use of certain prescribed medication	35 (28.0)	51 (40.8)	29 (23.2)	10 (8.0)	0.0

Question	n (%)				
	Always	**Often**	**Sometimes**	**Rarely**	**Never**
· Current Use of certain OTC medication	36 (28.8)	43 (34.4)	33 (26.4)	13 (10.4)	0.0
History of any experienced side effects related to the medication	28 (22.4)	42 (33.6)	37 (29.6)	16 (12.8)	2 (1.6)
Upon dispensing an OTC medication, do you provide counselling about?					
· Dosage regimen	110 (88.0)	15 (12.0)	0.0	0.0	0.0
· The proper storage conditions	60 (48)	43 (34.4)	14 (11.2)	8 (6.4)	0.0
· The shelf life post-opening	41 (32.8)	45 (36)	22 (17.6)	16 (12.8)	1 (0.8)
· The duration of medication use	65 (52.0)	39 (31.2)	16 (12.8)	5 (4.0)	0.0
· The proper administration method for (nebulizer, suppositories, etc.)	73 (58.4)	41 (32.8)	11 (8.8)	0.0	0.0
· The proper Indications for the OTC medication (such as antipyretic, vitamin, etc.)	93 (74.4)	26 (20.8)	4 (3.2)	2 (1.6)	0.0
· The effect administration regarding the meals (after eating, before eating)	94 (75.2)	28 (22.4)	3 (2.4)	0.0	0.0
When to stop using it and refer to the doctor	42 (33.6)	39 (31.2)	38 (30.4)	6 (4.8)	0.0
· Any specified precautions regarding drug-drug or drug-food interactions	46 (36.8)	57 (45.6)	20 (16.0)	2 (1.6)	0.0
·Any specified side effects that could the patient experience after using the medication and how to deal with it	32 (25.6)	40 (32.0)	36 (28.8)	15 (12.0)	2 (1.6)
Upon dispensing an OTC medication, what are the challenges that you counteract the proper pharmaceutical counselling process?					
· Limitations in counselling time	26 (20.8)	42 (33.6)	29 (23.2)	16 (12.8)	12 (9.6)
· Shortage in the drug related knowledge	2 (1.6)	27 (21.6)	36 (28.8)	35 (28.0)	25 (20.0)
· Shortage in the disease related knowledge	1 (0.8)	29 (23.2)	41 (32.8)	39 (31.2)	15 (12.0)
· Non-cooperated patients	8 (6.4)	26 (20.8)	49 (39.2)	26 (20.8)	16 (12.8)
· Dealing with patients under 18 years old	12 (9.6)	32 (25.6)	44 (35.2)	25 (20.0)	12 (9.6)
· Work overload, patients more than the pharmacist capacity	30 (24.0)	41 (32.8)	26 (20.8)	22 (17.6)	6 (4.8)
· Lack of counselling area	28 (22.4)	30 (24.0)	24 (19.2)	17 (13.6)	26 (20.8)

Table 3. The score of pharmacist perception for the importance of some counselling points (1-5, least -most important)

Counselling points	**Mean±STD**
· Explain the proper storage conditions of the medication	3.78±2.12
· Explain the proper duration of treatment and use of the medication	3.94±2.12
· Explain the contraindications of the medication	3.91±2.83
· Explain the proper administration method of the medication	3.98±2.83
· Explain the common side effects of the medication and how to deal with it	3.58±2.83
An overall score (out of 5)	**3.84±2.55**

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دور الصيادلة في تقديم المشورة للمرضى بشأن الأدوية التي لا تستلزم وصفة طبية في غير العاصمة: دراسة مقطعية

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ملخص

الخلفية: يمثل صيادلة المجتمع الخبراء الطبيين الأسهل للوصول إلى الأدوية. يلعبون دورًا رئيسيًا في تثقيف وإرشاد المرضى، خاصة فيما يتعلق بالأدوية التي لا تستلزم وصفة طبية.(OTC)

الغرض: هدفت هذه الدراسة إلى استكشاف دور الصيادلة في إرشاد المرضى للأدوية التي لا تستلزم وصفة طبية في العقبة، الأردن.

الطريقة: تم إجراء مسح مقطعي مع صيادلة المجتمع في العقبة. تم إطلاق استبيان عبر الإنترنت لعينة الدراسة عبر منصة التواصل الاجتماعي مثل (WhatsApp) تم استيراد الردود إلى الحزمة الإحصائية للعلوم الاجتماعية (SPSS) للتحليل الإحصائي.

النتائج: بدأ حوالي نصف الصيادلة استشاراتهم بالسؤال عن تاريخ المريض. نصح أكثر من 70 ٪ من الصيادلة المرضى من حيث نظام الجرعات، والإشارات المناسبة للأدوية التي لا تتطلب وصفة طبية، وأي تفاعلات محتملة بين الغذاء والدواء. ومع ذلك، أشار ما يقرب من ثلث الصيادلة إلى وجود العديد من التحديات في عملية الاستشارة. وشملت هذه القيود وقت الاستشارة، وعبء العمل الزائد، ومرضى أكثر من قدرة الصيدلي ونقص منطقة الاستشارة.

الخلاصة: توضح هذه الدراسة أن صيادلة المجتمع ملتزمون بشدة بمتابعة دورهم في الرعاية الصيدلانية من خلال تقديم المشورة المناسبة للأدوية التي لا تستلزم وصفة طبية في العقبة. علاوة على ذلك، تسلط دراستنا الضوء على بعض التحديات التي يمكن أن يواجهها الصيادلة، والتي قد تتداخل مع فعالية وسلامة الأدوية التي يقدمونها.

الكلمات الدالة: صيدلية المجتمع، الإرشاد، الأردن، الأدوية التي تصرف بدون وصفة طبية.

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Evaluation of phytochemical and pharmacological activities of *Taraxacum syriacum* and *Alchemilla arvensis*

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ABSTRACT

Oxidative stress, obesity, and multidrug resistance to pathogenic microorganisms are major challenges in the health care systems and pharmaceutical industries that prompt scientists to search for alternative sources with maximum efficacy and few side effects. Therefore, this study aimed to screen phytoconstituents, and estimate total phenols, flavonoids contents, antioxidant, antilipase, and antimicrobial activities of two selected plants, *Taraxacum syriacum* and *Alchemilla arvensis* four extracts. Conventional phytochemical assays were utilized for qualitative and quantitative determinations of the major phytochemical classes, total phenol, and flavonoids contents of methanol, hexane, acetone, and water extracts of both plants. While the antioxidant activity was evaluated using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. In addition, the antilipase activity was conducted using the porcine pancreatic lipase inhibitory test which was conducted by using a UV-visible spectrophotometer. Moreover, the antimicrobial activity of both plants' four extracts was established utilizing agar diffusion and micro-broth dilution methods against six microbial strains. The results revealed that the highest total phenol content was observed in the *T. syriacum* acetone extract (272.0 mg gallic acidE/g), while the highest total flavonoid content was detected in *A. arvensis* methanolic extract (83.3 mg rutinE/g). Actually, *T. syriacum* water extract has the best antioxidant potential among other extracts with an IC_{50} value of 95.5 µg/ml while *A. arvensis* acetone extract has the best antioxidant activity among other plant extracts with an IC_{50} dose of 4.9 µg/ml. Regarding antilipase activity, *A. arvensis* water extract showed a potent porcine pancreatic lipase inhibitory effect with an IC_{50} value of 21.4 µg/ml. However, most of the evaluated *T. syriacum* and *A. arvensis* plants' extracts showed broad-spectrum antibacterial and antifungal activities. This study recommended targeting these potentially medicinal plants in antioxidants and anticipate drugs for further *in-vivo* and preclinical studies.

Keywords: *Taraxacum syriacum; Alchemilla arvensis*; antioxidant; antilipase; antimicrobial.

INTRODUCTION

Plants provide endless sources of active therapeutic agents for the treatment of several diseases; in contrast to chemical medications which may be more expensive and more harmful [(1)]. Nowadays, many medical practitioners are looking at herbal remedies for common ailments, and these have gained momentum in the medical field, which are becoming more popular [(2; 3; 4)]

Taraxacum syriacum Boiss. is an annual herbaceous plant from the *Compositae* family [(5; 6)], whose roots have been used for treating hepatic diseases, anemia, gout, rheumatism, gastric ulcers and skin diseases, such as

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eczema. *T. syriacum* was used in traditional herbal medicine for the treatment of jaundice, liver disorders, and gallstones, as it lowering the total cholesterol, triglyceride, insulin, and fasting glucose levels, as well as insulin resistance induced by a high-fat diet. Therefore, *T. syriacum* extracts may show promise for both the prevention and treatment of fatty liver disease triggered by obesity**.** Morphologically, the plant has a rosette leaf arrangement with pinnate-type leaves and dentate leaf margins, although it does not have stipules. It has yellow flowers which appear in April [7].

Alchemilla arvensis (L.) Scop. belongs to the *Rosaceae* family and used to treat various health problems, such as kidney, bladder stones, renal edema and hepatic disorders. *A. arvensis* is an edible plant, particularly the leaves which are consumed as a salad. It has 2–20 cm in height with fan-shaped leaves. The flowers are less than 2 mm in length, occur in dense clusters in leaf-axils, surrounded by cups formed by leaf stipules, and bloom in March till mid of summer. Compositions of *Alchemilla* are rich in tannins and other minor components of therapeutic interest. It has been used in the treatment of diarrhea since it has important astringent properties. *A. arvensis* has also been used to treat stomach aches and gastrointestinal inflammation [8].

Nowadays, free radicals, obesity, and microbial resistance to antibiotics represented serious health care problems. For that, many studies were established to solve these complexities by providing natural herbal plants medicine and cure forms. Thus, this study was aimed to screen phytochemical constituents of two recommended medicinal plants (*A. arvensis* and *T. syriacum),* to estimate their potential total phenols and flavonoids contents as well as to assess their antioxidant, anti-lipase, and antimicrobial properties.

MATERIALS AND METHODS

Collection and identification of plant materials

The roots of *T. syriacum* and leaves of *A. arvensis* were collected in April 2017 from the Nablus region in Palestine. The collected plants were identified by An-Najah National University experts before they were air-dried at room temperature for later use.

Extraction of plant materials

The air-dried plant materials (leaves and roots) were ground to a uniform powder, to be used for two extractions: organic and aqueous (crude) ones. Organic extraction was performed using the Soxhlet extraction method [9]. This extraction was established by taking 20 g of dried plant powders in a glass thimble using 250 ml of each solvent separately (methanol and acetone). The extraction processing was continued till the solvent in the siphon tube of the Soxhlet apparatus became colorless. Then extracts were incubated in a hot water bath at 35ºC until the solvent had completely evaporated. The dried plant crude extract was stored in the refrigerator at 4ºC for later use. While, the aqueous extraction was performed from 5 g of the plant powder that mixed with 200ml of distilled water and then heated to reach 30–40ºC with continuous stirring for 20 min [10]. The mixture was filtered using Whatman filter paper and used for phytochemical analysis.

Phytochemical screening

Phytochemical screenings were performed using standard procedures according to the methods reported by Trease and Evans [11].

Phenols and flavonoids tests

Total phenolic contents (TPC) in plant extracts were determined using a spectrophotometric method with some modifications [12]. The aqueous solutions of methanolic extracts (1 mg/ml) were prepared for the analysis. The reaction mixture was prepared by mixing 0.5 ml of plant extract solution, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 ml of 7.5% of $NaHCO_3$ aqueous solution. Samples were incubated in a thermostat at 45ºC for 45 min. The absorbance was determined by a

spectrophotometer at 765 nm wavelength. The samples were prepared in triplicate for each analysis and the mean value of absorbance was reported. The same procedure was repeated for the standard solution of gallic acid and a calibration curve was constructed. Based on the measured absorbance, the concentration of phenol content was expressed in terms of gallic acid equivalent (mg GAE/g).

Total flavonoid content (TFC) was determined from the calibration curve of rutin (reference substance) and was expressed as milligram of rutin equivalent per gram of extract (mg RUE/g extract) [13]. Total flavonoid content was determined according to the modified procedure of Chang *et al.* [13], and validated by Nugroho *et al*. [14]. Rutin (100 mg) was dissolved in 10 ml distilled water and diluted in a final volume of 100 ml. Subsequently, the stock solution was diluted to provide a series of concentrations (5, 10, 20, 40, and 100 mg/ml). Aliquots of each solution (0.5 ml) were mixed with 3 ml methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1 M potassium acetate, and 5 ml distilled water and then incubated at room temperature for 30 min. Absorbance was measured at 415 nm wavelength using a spectrophotometer. Distilled water with methanol, 10% $AlCl_3$, and potassium acetate were used as a blank. The total flavonoids contents of both screened plants extracts were expressed as rutin equivalents (mg of RUE/g plant extract).

Antioxidant DPPH- test

Stock solutions of plant extract and Trolox (the reference substance) were prepared at a concentration of 0.1 mg/ml in methanol. Working solutions at concentrations of 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, and 100 µg/ml were prepared by serial dilution of the respective stock solution in methanol.

DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above-prepared working solutions in a ratio of 1:1:1, respectively. Methanol was used as a blank solution. The first solution of the series concentration was DPPH with methanol only. The solutions were incubated in dark for 30 min at room temperature before the absorbance readings were recorded at 517 nm [15]. The percentage of antioxidant activity of the plants and the Trolox standard was calculated using the following formula:

DPPH activity (%) = (A-B)/A ×100

where A = optical density of the blank, and B = optical density of the sample.

Antilipase test

The porcine pancreatic lipase inhibitory assay was adapted from the published method of Zheng *et al*. [16], with some modifications. Plant extract stock solution (1 mg/ml) was used to prepare five different solutions in 10% DMSO at concentrations of 200, 400, 600, 800 and 1000 µg/ml. A stock solution of pancreatic lipase enzyme (Riedeldehan, Germany) (1 mg/ml) in Tris-HCl buffer was prepared immediately before use.

A stock solution of *p*-nitrophenyl butyrate (PNPB) was prepared by dissolving 20.9 mg in 2 ml of acetonitrile. For each working test tube, 0.1 ml of porcine pancreatic lipase (1 mg/ml) was added to a test tube containing 0.2 ml plant extract from each diluted solution series for each studied plant. The resulting mixture was then made up to 1ml by adding Tris-HCl solution and was incubated at 37°C for 15 min. After the incubation period, 0.1 ml of PNPB solution was added to each test tube. The mixture was incubated for a further 30 min at 37°C.

Pancreatic lipase inhibitory activity was determined by measuring the hydrolysis of p-nitrophenolate to p-nitrophenol at 405 nm using a spectrophotometer. The same procedure was repeated for Orlistat which was used as a positive control.

Antimicrobial assays

Antibacterial test

Antibacterial activity was tested by simple agar diffusion [17], against *Staphylococcus aureus* [ATCC 25923], *Escherichia coli* [ATCC 25922] *Pseudomonas aeruginosa* [ATCC 27853], and *Shigella sonnie* [ATCC 25931]. Agar media plates were gently swabbed with

turbidity-adjusted bacterial suspension before 50 µl of plant extracts were added to the wells. Plant extracts were obtained by different extraction methods (methanol, acetone, hexane, and aqueous crude extracts) and dissolved in 1 ml of 10% DMSO, before filling agar gel wells. Plates were then incubated for 16–18 h at 37°C and the antibacterial activity was evaluated by measuring the diameter of clear zones surrounding the wells.

The minimal inhibitory concentration (MIC) of the plant extracts against bacteria was determined using the micro-broth dilution method [18]. Mueller-Hinton broth (MHB) was used for this test in a polystyrene panel containing approximately 96 wells including a positive and negative growth control. The plant extract (100 µl) was added to the first wells, which already contained 100 µl MHB and then serially diluted with MHB in the remaining wells. The microorganisms (bacteria) were added to the MHB in a tube using a loop at a concentration of 1.5 $\times 10^8$ CFU/ml, compared to McFarland standard. The bacterial suspension was then diluted 1:3 with 4ml MHB to a concentration of 5×10^7 CFU/ml. Then the bacterial suspension (1 µl) was applied to all wells except the negative control one. The panel was covered and incubated at 35˚C for 16–20 hrs.

Several antibiotics (Azithromycin; Clarithromycin; Levofloxacine; Doxycycline; Cefuroxime; and Ciprofloxacin (Birzeit Pharmaceutical company, Palestine) were used to determine bacterial resistance. These were dissolved in their corresponding solvents according to the solubility tests; and prepared as a 1:10 dilution.

Antifungal test

Antifungal activity was examined against two pathogenic fungi *Epidermophyton floccosum* [ATCC52066] and *Candida albicans* [ATCC 90028] that were cultured on Potato Dextrose Agar (PDA) media. The antifungal activity of plant extracts against *Candida albicans* was determined using the micro-broth dilution method, similar to the previously reported procedure for MIC determination of bacterial isolate, with some modifications in inoculums preparation [19]. The *Candida* concentration in a broth with turbidity similar to 0.5 McFarland was $1\times10^6$to 5×10^6CFU\ml. This was diluted twice, 1:50 and 1:20, first in MHB and then in RPMI media, resulting in 1×10^3 to 5×10^3CFU/ml before the aliquots of (100 µl) were added to each well containing a defined concentration of plant extract. On the other hand, the antifungal activity against *Epidermophyton floccosum* was determined using the agar dilution method [20]. In this method, the plant extract was serially diluted with Sabouraud's Dextrose Agar (SDA). The fungus was prepared by adding sterile distilled water with 0.05% Tween 80 onto the surface growth. Spores and hyphae were then scraped off using a sterile scalpel. The turbidity of the resulting suspension was adjusted to be equivalent to 0.5 McFarland (absorption 0.08 to 1 at 600 nm). Later, 20 µl of the fungal solution was added to each tube and incubated at 25 ºC for 14 days. MIC was measuring the lowest concentration of plant extract that caused visible inhibition of fungal growth. Two types of antifungal drugs (Terbenafin and Tinidazole); which mixed in their suitable solvents, were diluted in a 1:10 dilution and used to determine the resistance of fungi [21].

RESULTS & DISCUSSION

Phytochemical screening

The two selected medicinal plants (*T. syriacum* and *A. arvensis*) (Figure 1) were collected from Palestinian territories and subjected to four different extraction methods.

(a) **(b)** **(c)**

Figure 1: The collected plants *Taraxacum syriacum* (a,b) and *Alchemilla arvensis* (c) from Palestinian territories.

Medicinal plants are containing phytochemical compounds include tannins, alkaloids, carbohydrates, terpenoids, steroids, and flavonoids. They are widely used in human therapy, veterinary, agriculture, and scientific research. There are large numbers of phytochemicals belonging to chemical classes, which have been shown to have inhibitory effects on many types of microorganisms in vitro.

Standard phytochemical tests were utilized to screen *T. syriacum* and *A. arvensis* four extracts. The results showed that *T. syriacum* methanolic and acetone extracts contained phenols, tannins, flavonoids, glycosides, and steroids. While hexane extract contains only cardiac glycosides. Moreover, the water extract contained phenols, tannins, flavonoids, and cardiac glycosides. In addition, *A. arvensis* methanolic extract contained phenols, tannins, glycosides, steroids, and cardiac glycosides. In fact, the results showed that the hexane extract did not contain any of screened phytochemicals while the acetone extract contained glycosides, steroids, and cardiac glycosides. The aqueous extract was containing only flavonoids.

Methanol extract yield was found the best one for phytochemical extraction from *T. syriacum* (12.5%) while water extract was the best yield for *A. arvensis* (25.6%) as shown in Table 1. Nine phytochemical tests were used to determine the presence of organic materials and the results revealed that *T. syriacum* and *A. arvensis* were rich in phytochemicals as presented in Table 2.

Total phenol and flavonoid contents

Phenolic compounds are reactive metabolites in a wide range of plant-derived foods and work as chelators of metal ions that are capable of catalyzing lipid oxidation. Besides, they have many beneficial properties, such as antioxidant effects, and anti-mutagenic activities, as well as they, can prevent cardiovascular diseases.

The total phenol and flavonoid contents were calculated in 1 g of plant extract (Table 3 and 4). Total phenol content was higher (272 mg GAE/g plant extract) in *T. syriacum* than in *A. arvensis* (151.5 mg GAE/g plant extract). In this study, *A. arvensis* revealed high total phenol content in the methanolic extract (151.51 mg GAE/g) compared with a similar one conducted by Kiselova *et al.*. [(22)], who found the total polyphenolic content between 88.00 and 112.33 µg/ml. In *T. syriacum,* the highest content of phenol was detected in acetone extract, equal to 271.95 mg GAE/g followed by water and methanol plant extracts with total phenolic contents of 143.7 and 120.4 mg GAE/g of plant extract. To the best of our knowledge, no previous studies have been conducted on total phenolic contents of *T. syriacum*.

Flavonoids are used in the treatment of eczema such as

quercetin. They also influence heart disease by reducing the risk of developing atherosclerosis by increasing the release of nitric oxide causing vasodilation. Also, they reduce allergic responses and stimulate the immune system.

This study revealed that flavonoid content was higher in *A. arvensis* (83.3 mg of RUE/g plant extract) than in *T. syriacum* (27.1 mg RUE/g plant extract). Aqueous extracts of *T. syriacum* were found to contain a high quantity of flavonoids (27.13 mg RUE/g plant extract), followed by acetone and methanolic extracts with total flavonoids contents of 17.8 and 8.4 mg RUE/g plant extract. To the best of our knowledge, no previous studies have been conducted on the total flavonoid contents of the *T. syriacum* plant. A study by Liu *et al.* [23] found that the total flavonoids content in *Taraxacum mongolicum* was 20.57±1.12 mg/g in methanol extract and 6.55±1.20 mg/g in water extract [23]. Aqueous extract of *A. arvensis* contained a high quantity of flavonoid and in 1 g of plant water extract, the quantity was 83.31 mg.

Antioxidant test-DPPH

Using DPPH assay, the antioxidant IC_{50} was calculated for each plant while the potent antioxidant drug Trolox was used as a positive control. In this study, *A. arvensis* has a potential antioxidant effect in methanol, hexane, and acetone extracts with IC_{50} values of 97.72, 11.22, and 4.86 µg/ml, respectively. These results showed that *A. arvensis* acetone extract had the most potent antioxidant effect among other plant extracts, While, *T. syriacum* aqueous extract has the highest antioxidant potential with an IC_{50} dose of 95.5 µg/ml among other plant extracts (Table 5).

In fact, the acetone extract exhibited potent antioxidant activity compared with Trolox which has an antioxidant IC_{50} value of 2.2 µg/ml. A study conducted by Nedyalkov *et al.* [24] found that *Alchemilla mollis* has also potent antioxidant capacity which was determined using four different assays (FRAP, CUPRAC, DPPH and ABTS).

Regarding, *T. syriacum* antioxidant activity, the best was detected in water extract which has an antioxidant IC_{50} dose of 95.49 µg/ml.

Antioxidants are bioactive molecules that inhibit the oxidation of free radicals which lead to chain reactions that can cause damage to cells and organs [25; 26].

Anti-lipase activity

The anti-lipase activity was detected using porcine pancreatic lipase inhibitory assay while Orlistat anti-obesity medication was utilized as a positive control. Table 6 depicts that both studied plants have an anti-lipase effect.

The results of the current study showed that water, methanol, acetone, and hexane extracts of *A. arvensis* had the highest antilipase activity with IC_{50} values of 21.37, 30.90, 45.70, and 72.44 µg/ml, respectively. To the best of our knowledge, no previous studies had been conducted on *A. arvensis* antilipase effect. Even though, a study of *Alchemilla vulgaris* was reported to show the inhibitory activity of pancreatic lipase [27; 28].

In *T. syriacum* plant, the best antilipase effect was shown in the water and hexane extracts, with IC_{50} values of 154.88 and 218.77 µg\ml, respectively. While the used positive control Orlistat has antilipase effect win an IC_{50} value of 20.4 µg\ml. To the best of our knowledge, no previous studies have been conducted on the *T. syriacum* as an antilipase agent.

Orlistat, as a lipase inhibitor, was used in this study as a positive control. The lipase inhibitors that are used to reduce the activity of lipases found in the intestine prevent the hydrolysis of dietary triglycerides to monoglycerides and fatty acids, so no absorption takes place. This mechanism could be used for the treatment of obesity [29].

Antimicrobial activity

Antimicrobials are medicinal products that kill or inhibit the growth of living microorganisms, usually called antibiotics because they act against bacterial infections [30]. These also include antimycobacterial, antiviral, antifungal, and antiparasitic drugs. Some bacteria are resistant to certain antibiotics and others can acquire resistance

through mutations in some of their genes when they are exposed to an antibiotic.

Bacteria causing a wide range of infections may become resistant to one or many antibiotics, such as those causing urinary tract infection, pneumonia, skin infection, and bloodstream infections. For example, a high proportion of resistance to third-generation cephalosporins was reported for *E. coli* and *K. pneumonia*.

Antibacterial effects of *Taraxacum syriacum* and *Alchemilla arvensis* plants were firstly verified with simple agar diffusion tests as shown in Table 7. The MIC values were also determined for each plant extract against each different bacteria, as shown in Table 8. Besides, drug resistances were tested for these selected bacteria with different antibiotics to reveal that Cefuroxime 250 mg had a low MIC value (2.35 µg/ml) for all tested bacteria; meanwhile, the most inhibitory antibiotic was recorded for Levofloxacin 500 mg and Ciprofloxacin (< 10 ng/ml).

The MIC values for different plant extracts against two types of fungi were determined as shown in Table 9. In addition, drug resistance was tested for these selected fungi against different antifungals to found that Terbinafine 250 mg has advantages in killing *C. albicans* over Tinidazole 500 mg

This study showed that *A. arvensis*, had antimicrobial effects for bacteria and fungi in different extracts, and MIC values for different types of bacteria and fungi as shown previously. To the best of our knowledge, no previous studies were conducted on *A.arvensis*. These results were in accordance with other ones on *Alchemilla vulgaris* [31] and *Alchemilla mollis* [32; 33] which resulted in presence of antibacterial activity in these plants.

Taraxacum syriacum has an antimicrobial effect on bacteria and fungi in different extracts, with different MIC values. To the best of our knowledge, no previous studies were conducted on *T. syriacum* antimicrobial effects. Even though studies on *Taraxacum mongolicum* [34] and *Taraxacum officinale* [35] revealed strong antimicrobial activity in vitro.

Finally; this study was the first to report the antioxidant, antilipase, antibacterial and antifungal activities of the *Taraxacum syriacum* and *Alchemilla arvensis* herbal plants; as well the total phenols and total flavonoids constituents that were not estimated before.

CONCLUSION

The phytochemical constituents, total phenols, and flavonoids contents of *A. arvensis* and *T. syriacum* were successfully reported in the current study. Both plants expressed high potentiality in their antioxidant, anti-lipase, and antimicrobial tests. The obtained results recommended the use of *A. arvensis* and *T. syriacum,* for the treatment of oxidative stress, obesity, and infectious diseases. Further phytochemical and pharmacological studies on *T. syriacum* and *A.arvensis* are required to isolate the bioactive compounds from these plants, elucidate their chemical structures and estimate their therapeutic activity *in vivo* besides investigating their toxicity and side effects.

Acknowledgment

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Table 1: The percentage yields of selected herbs from each different extraction solvents

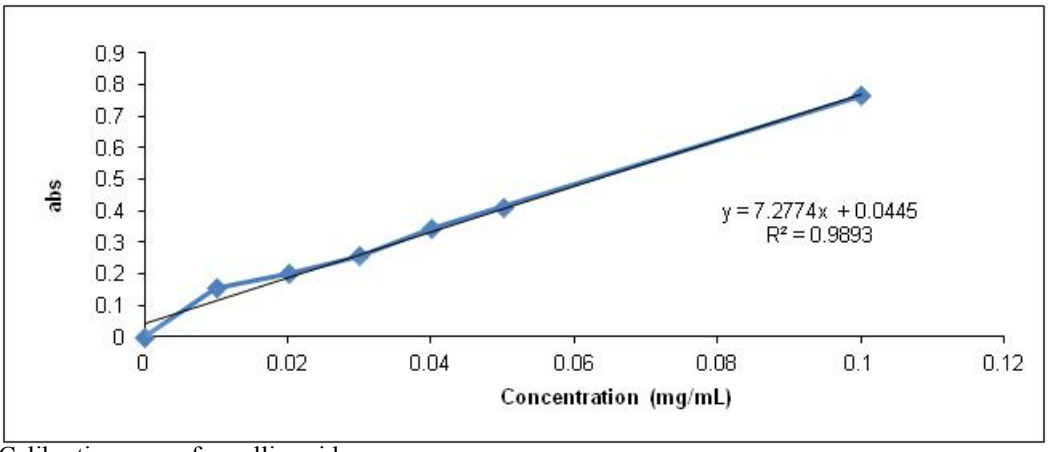
Extraction Method	Yields	
	T. syriacum	***A. arvensis***
Methanol	**12.5%**	14.2%
Hexane	2.07%	3.9%
Acetone	3.2%	2.8%
Water	2.2%	**25.6%**

Table 2: Phytochemical screening test results for *T. syriacum* and *A. arvensis*

Taraxacum syriacum				
Test \ Extract solvent	**Methanol**	**Hexane**	**Acetone**	**Water**
1. Protein (Millon's test)/(Ninhydrine)	-	-	-	-
2. Carbohydrate tests (Fehling's)/(Benedict's) /(Iodine)	-	-	-	-
3. Phenol and tannin ($FeCl_3$)	+	-	+	+
4. Flavonoid (Shinoda) /(Alkaline)	+	-	+	+
5. Saponins	-	-	-	-
6. Glycosides (Liebermann's) /(Salkowski's)	+	-	+	-
7. Cardiac steroidal glycoside	-	+	-	+
8. Steroid	+	-	+	-
9. Terpenoids	-	-	-	-
Alchemilla arvensis				
Test \ Extract solvent	**Methanol**	**Hexane**	**Acetone**	**Water**
1. Protein (Millon's test)/(Ninhydrine)	-	-	-	-
2. Carbohydrate (Fehling's)\ (Benedict's)\(Iodine)	-	-	-	-
3. Phenol and tannin ($FeCl_3$)	+	-	-	-
4. Flavonoid (Shinoda) /(Alkaline)	-	-	-	+
5. Saponins	-	-	-	-
6. Glycosides (Liebermann's)/(Salkowski's)	+	-	+	-
7. Cardiac steroidal glycoside	+	-	+	-
8. Steroid	+	-	+	-
9. Terpenoids	-	-	-	-

Table 3: Total phenol content results.

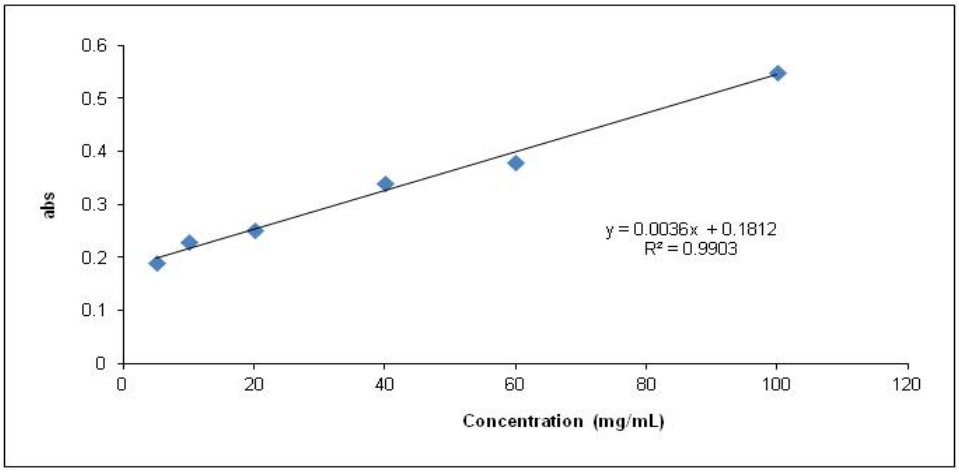
Plant	Extract solvent	Total phenol content (mg GAE/g)
T. syriacum	Methanol	120.4
	Acetone	272.0
	Water	143.7
A. arvensis:	Methanol	151.5



Calibration curve for gallic acid

Table 4: Total flavonoid content in *T. syriacum* and *A. arvensis* plants

Plant	**extract solvent**	**Total flavonoid content (**RUE/g plant extract**)**
T. syriacum	Methanol	8.4
	Acetone	17.8
	Water	27.1
A. arvensis	Methanol	83.3



Calibration curve for rutin

Table 5: Antioxidant IC_{50} values for *T. syriacum* and *A. arvensis* and DPPH inhibitory activity curves by both plants

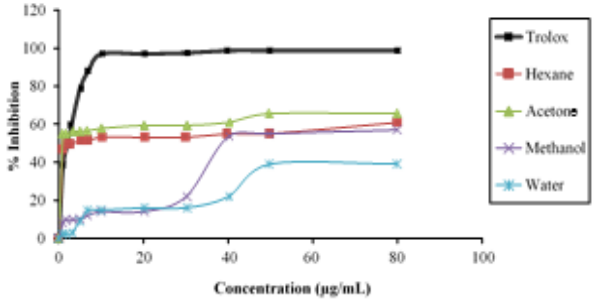
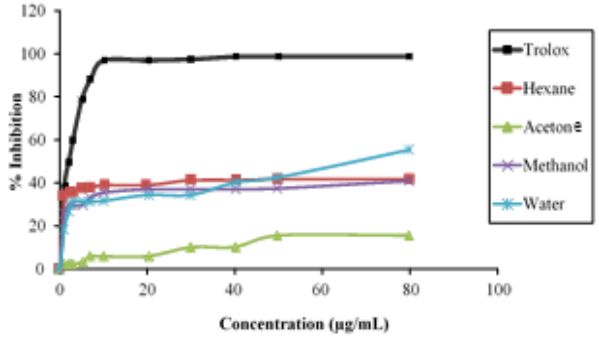
Plant	Extracts	IC_{50} (µg/ml)	
T. syriacum			
	Hexane	177.8	
	Methanol	281.8	
	Acetone	7079457.8	
	Water	95.5	
			DPPH inhibitory activity curves by *T. syriacum*
A. arvensis			
	Hexane	11.2	
	Methanol	97.7	
	Acetone	4.9	
	Water	724.4	
			DPPH inhibitory activity curves by *A. arvensis*
	Trolox	2.2	

Table 6: Antilipase IC_{50} values of *T. syriacum and A. arvensis and* porcine lipase inhibitory curve by both plants

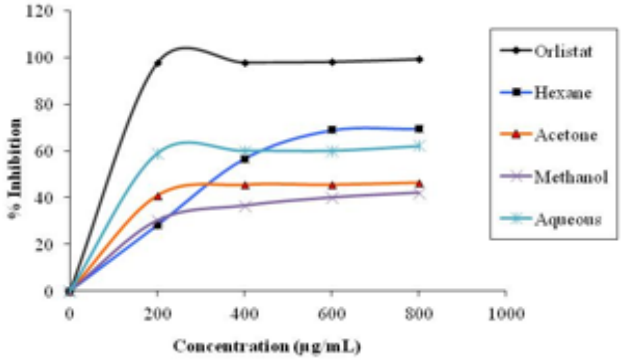
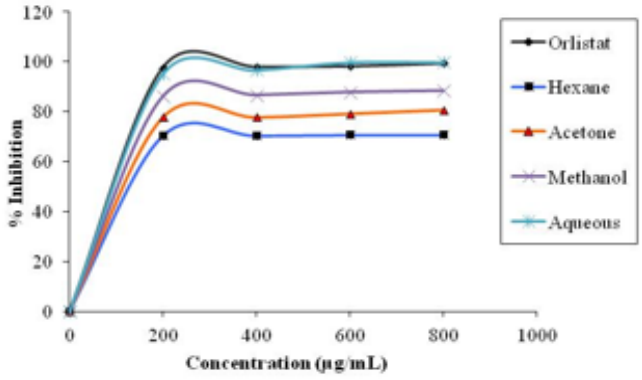
Plant	Extracts	IC_{50} (µg/ml)	
T. syriacum			
	Hexane	218.8	
	Methanol	3311.3	
	Acetone	977.2	
	Water	154.9	Antilipase curve for *T. syriacum*
A. arvensis			
	Hexane	72.4	
	Methanol	30.9	
	Acetone	45.7	
	Water	21.4	Antilipase curve for *A. arvensis*
	Orlistat	20.4	

Table 7: Results of simple agar diffusion method

T. syriacum plant					
	Plant extract solvent	**Water**	**Acetone**	**Methanol**	**Hexane**
	Bacteria name	**Inhibition zone diameter (mm)**			
	Escherichia coli	-	-	-	-
	Pseudomonas aeruginosa	-	10	-	-
	Staphylococcus aureus	-	-	-	10
	Shigella sonnie	-	-	-	-
A. arvensis plant					
	Escherichia coli	20	10	10	-
	Pseudomonas aeruginosa	-	-	-	-
	Staphylococcus aureus	15	10	10	-
	Shigella sonnie	-	20	15	10

Table 8: MIC values for *T. syriacum* and *A. arvensis* with bacteria

T. syriacum plant						
	Plant extract solvent	**Water**	**Acetone**	**Methanol**	**Hexane**	**DMSO**
	Bacterial Name	**MIC values (mg/ml)**				
	Staphylococcus aureus	6.3	12.5	12.5	No inhibition	6.3
	Pseudomonas aeruginosa	25	12.5	6.3	No inhibition	6.3
	Escherichia coli	25	12.5	6.3	No inhibition	6.3
	Shigella sonnie	25	12.5	12.5	No inhibition	6.3
A. arvensis plant						
	Staphylococcus aureus	25	25	3.1	No inhibition	6.3
	Pseudomonas aeruginosa	12.5	No inhibition	3.1	No inhibition	6.3
	Escherichia coli	12.5	No inhibition	3.1	No inhibition	6.3
	Shigella sonnie	12.5	25	3.1	12.5	6.3

Table 9: MIC values for *T. syriacum*, and *A. arvensis* with fungi

T. syriacum plant						
	Plant extract solvent	**Water**	**Acetone**	**Methanol**	**Hexane**	**DMSO**
	Fungus Name	**MIC values (mg/ml)**				
	Candida albicans	25	3.1	6.3	6.3	3.7
	Epidermophyton floccosum	1.6	0.8	0.8	0.8	6.3
A.arvensis plant						
	Candida albicans	12.5	6.3	6.3	12.5	3.7
	Epidermophyton floccosum	0.78	0.78	0.78	0.78	6.3

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تقييم النشاط الكيميائي النباتى والدوائى لنباتين طبيين (*Alchemilla arvensis* و *Taraxacum syriacum*)

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ملخص

يعد الإجهاد التأكسدي والسمنة ومقاومة الأدوية المتعددة للكائنات الدقيقة المسببة للأمراض من التحديات الرئيسية في أنظمة الرعاية الصحية والصناعات الدوائية والتي تدفع العلماء للبحث عن مصادر بديلة بأقصى قدر من الفعالية وآثار جانبية قليلة. لذلك هدفت هذه الدراسة إلى فحص المكونات النباتية وتقدير إجمالي محتوى الفينولات والفلافونويدات ومضادات الأكسدة ومضادات اللايبيز والأنشطة المضادة للميكروبات ل أربعة مستخلصات من نباتين مختارين هما Taraxacum syriacum و Alchemilla arvensis. تم استخدام فحوصات كيميائية نباتية تقليدية للتحديد النوعي والكمي للفئات الكيميائية النباتية الرئيسية، ومحتوى الفينول الكلي، ومحتويات الفلافونويد من الميثانول، والهكسان، والأسيتون، والمستخلصات المائية لكلا النباتين. بينما تم تقييم نشاط مضادات الأكسدة باستخدام مقايسة DPPH (2،2-Diphenyl-1-picrylhydrazyl). . بالإضافة إلى ذلك، تم إجراء نشاط مضادات اللايبيز باستخدام اختبار مثبط لايبيز بنكرياس الخنازير والذي تم إجراؤه باستخدام مقياس الطيف الضوئي المرئي للأشعة فوق البنفسجية. علاوة على ذلك، تم إنشاء النشاط المضاد للميكروبات للمستخلصات الأربعة للنباتين باستخدام طريقة الانتشار بالأجار وطرق التخفيف الدقيق للبيئة المغذية ضد ستة سلالات ميكروبية. أظهرت النتائج أن أعلى محتوى إجمالي للفينول لوحظ في مستخلص T. syriacum acetone (272.0 مجم حمض جاليك / جم)، بينما تم الكشف عن أعلى محتوى إجمالي من الفلافونويد في مستخلص A. arvensis الميثانولي (83.3 مجم روتين إي / جم). في الواقع، يحتوي المستخلص المائي من T. syriacum على أفضل إمكانات مضادات الأكسدة من بين المستخلصات الأخرى بقيمة IC50 تبلغ 95.5 ميكروغرام / مل بينما يحتوي المستخلص الاسيتوني من A. arvensis على أفضل نشاط مضاد للأكسدة من بين المستخلصات النباتية الأخرى بجرعة IC50 تبلغ 4.9 ميكروغرام / مل. فيما يتعلق بنشاط مضادات اللايبيز، أظهر المستخلص المائي من A. arvensis تأثيرا مثبطا قويا للايبيز بنكرياس الخنازير بقيمة IC50 بقيمة 21.4 ميكروغرام / مل. ومع ذلك، أظهرت معظم مستخلصات نباتات T. syriacum و A. arvensis التي تم تقييمها أنشطة واسعة النطاق كمضادات للبكتيريا والفطريات. أوصت هذه الدراسة باستهداف هذه النباتات الطبية المحتملة في مضادات الأكسدة والأدوية الاستباقية ولمزيد من الدراسات على الجسم الحي وما قبل السريرية.

الكلمات الدالة: Taraxacum syriacum؛ Alchemilla arvensis، مضادات الأكسدة، مضادات اللايبيز، مضادات الميكروبات

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Development of Novel HPLC Method for Analysing Drugs Used in H-Pylori Treatment

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ABSTRACT

Helicobacter pylori (H. pylori) is the most chronic bacterial infection on human being that is found in the gastric mucous layer and adapted to survive in acidic conditions. Because of the resistance of this organism, triple therapy treatment is required. In this study, RP-HPLC method was developed in terms of mobile phase composition, buffer concentration and additive amount to separate the complex drugs mixture used as triple therapy for H. pylori treatment. The first result showed that C18 reversed phase column has better resolution than C8 for analyzing amoxicillin, metronidazole, omeprazole and clarithromycin at isocratic elution mode. Design of experiments was then implemented to evaluate the best separation parameters. The results showed that amoxicillin was detected at 254 nm, where metronidazole, omeprazole and clarithromycin were detected at 304 nm. Moreover, better resolution was achieved at mobile phase compositions of 30:30:40 (acetonitrile: methanol: buffer), respectively. Regarding the optimum amount of trimethylamine added to the mobile phase to improve the resolution, the outcomes showed that 30 µL was the best choice at pH around 6.0 with 0.05 M potassium dihydrogen phosphate as a buffer. The developed method could separate the mixture as following; amoxicillin and omeprazole at 2.56, 4.84, respectively, where metronidazole and clarithromycin were retained 3.04 min.

Keywords: RP-HPLC, Design of Experiments, Triple therapy, H. pylori.

1. INTRODUCTION

H. pylori is the most chronic bacterial infection that is found in the gastric mucous layer and adapted to survive in acidic conditions inside the human body [1-3]. Because of the resistance of these bacteria, multidrug therapy is required [4]. H. pylori is responsible for gastric and duodenal ulcer disease that infects approximately 50% of the human population worldwide [5-6]. The predominant shape of this organism is curve or spiral [7-8] as shown in Figure.1. Peptic ulcers are localized erosion of the mucous membrane with a diameter of at least 0.5 cm of the stomach and duodenum [9]. The main causes of peptic ulcer disease are multifactorial such as Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) or aspirin, alcohol and tobacco consumption [10], however, H. pylori is the one of the most important causes [11]. The pain related to ulcer is caused by irritation of exposed surfaces by the gastric acids [12].

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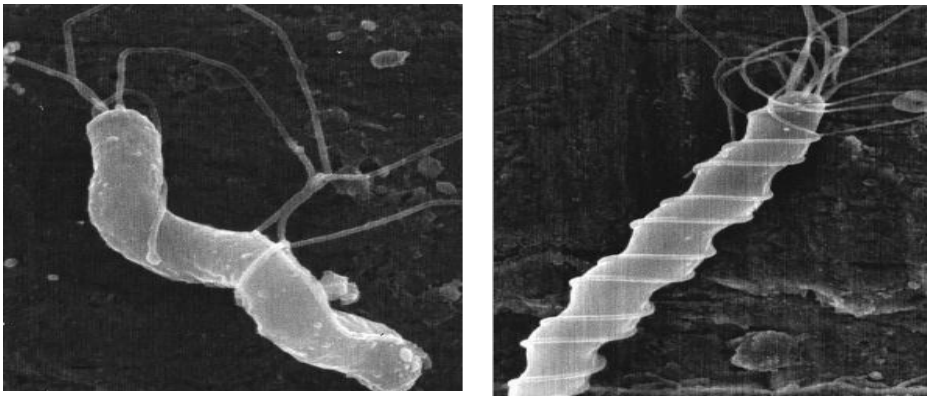

Figure.1. The predominant shape of H. pylori

The common medical treatment for ulcer caused by H. pylori requires a combination of at least three drugs (i.e. triple therapy). The recommended triple therapy consists of proton pump inhibitors (PPIs), mainly; omeprazole combined with two antibiotics such as: clarithromycin and amoxicillin or clarithromycin and metronidazole [12-13] with a time range of 10- 14 day [4]. Omeprazole (Figure 2-B) consists of 2-pyridyl methyl sulfinyl benzimidazoles with different substitutions on the pyridine or the benzimidazole [14-15]. It is considered as a weak base; hence, it can be used to regulate the acid production in the stomach [16-18]. Omeprazole inhibits the production of gastric acid by blocking the hydrogen-potassium adenosine triphosphate enzyme system (H^+, K^+ and the ATPase) (i.e. the proton pump which is essential for acid secretion by parietal cells that is the most acidic cell in the body) [19-20].

Metronidazole (Figure 2) belongs to a class of drugs called nitroimidazole that contains an imidazole ring [21]. In vivo, the NO_2 group on position 5 in metronidazole is reduced by bacteria and protozoan [22-23]. The reduction product is responsible for antimicrobial activity of metronidazole [22, 24-25]. Regarding clarithromycin (Figure 2), it belongs to a class of drug called macrolide that derived from erythromycin [26]. It is 6-O methyl erythromycin with a 14-membered macrocyclic lactone ring attached to two deoxy sugars (cladinose and desosamine) [27-28]. It is an acid stable antibiotic and well absorbed from the intestines where it shows an excellent antimicrobial activity that penetrates the stomach tissue [29-30]. Amoxicillin (Figure 2-D) is considered as an antibiotic derived from penicillin, 6-aminopenicillanic acid [31]. The chemical structures of the triple therapy drugs are shown in Figure 2, the chemical and physical properties are shown in Table 1.

Table 1. Chemical and physical properties of omeprazole, metronidazole, clarithromycin, and amoxicillin

Name of the drug	Omeprazole	Metronidazole	Clarithromycin	Amoxicillin
Chemical formula	$C_{17}H_{19}N_3O_3S$	$C_6H_9N_3O_3$	$C_{38}H_{69}NO_{13}$	$C_{16}H_{19}N_3O_5S$
Molar mass (g/mol)	345.42	171.15	747.95	419.64
Storage conditions	2-8 ºC	Room temperature	-20° C	2-8°C
Appearance	White powder	White powder	Off-White powder	White powder
Log P	2.23 [32]	-0.02 [33]	3.16 [35]	0.87 [36]

pKa	4.77 [32] 9.29	2.38 [34]	8.99 [35]	2.67 [36] 7.11 9.55
Solubility	Dissolve at alkaline pH. Soluble in methanol and has solubility in water of 0.52 mg/mL at 25°C.	Slightly soluble in methanol and has a solubility in water of 5.92 mg/mL at 25°C.	Slightly soluble in methanol and has a solubility in water of 0.22 mg/mL at 25°C.	Slightly soluble in methanol and has solubility in water of 3.4 mg/mL at 25°C.
Name of the drug	Omeprazole	Metronidazole	Clarithromycin	Amoxicillin
Chemical formula	$C_{17}H_{19}N_3O_3S$	$C_6H_9N_3O_3$	$C_{38}H_{69}NO_{13}$	$C_{16}H_{19}N_3O_5S$
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Solubility	Dissolve at alkaline pH. Soluble in methanol and has solubility in water of 0.52 mg/mL at 25°C.	Slightly soluble in methanol and has a solubility in water of 5.92 mg/mL at 25°C.	Slightly soluble in methanol and has a solubility in water of 0.22 mg/mL at 25°C.	Slightly soluble in methanol and has solubility in water of 3.4 mg/mL at 25°C.

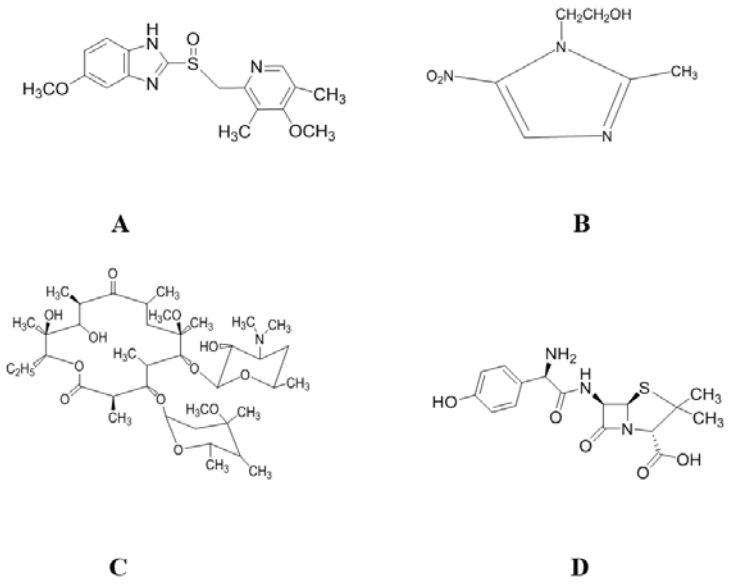

Figure 2. Chemical structures of: A) omeprazole, B) metronidazole, C) clarithromycin, and D) amoxicillin

High Performance Liquid Chromatography (HPLC) is an important tool for quantitative and qualitative analysis in pharmaceutical field [37-39]. Moreover, reversed phase high performance liquid chromatography (RP-HPLC) has been used in a wide range of separation in the field of pharmaceutical analysis, biology, proteomics and metabolomics [40-41], petroleum industry [41-42] and environmental analysis [41, 43], due to the wide range of commercial columns available.

Many studies reveal different methods for separating two or three of triple therapy drugs in a mixture using chromatographic techniques, where analytical methods have been developed for the quantitative analysis of these compounds, either alone or with combination with other drugs [31, 44-54]. For example, Walash et al., [49] developed new HPLC method for the determination of a ternary mixture of omeprazole, tinidazole and doxycycline in their raw materials and combined capsules using methanol and sodium dihydrogen phosphate at pH 4.5 using C18 column with retention time less than 6 min. El-khoudary et al., [51] used gradient elution in RP-HPLC to separate clarithromycin, norfloxacin, doxycycline, tinidazole and omeprazole in less than 9 min using ion pairing agent, phosphate buffer, and octanesulphonic acid with acetonitrile as a mobile phase. Moreover, Darwish et al., [48] also implemented C18 column to analyze tinidazole, doxycycline, omeprazole and clarithromycin within less than 10 min in a gradient elution mode using acetonitrile, methanol and potassium dihydrogen phosphate. Another study used C8 column for the separation of omeprazole, tinidazole and clarithromycin [52] in bulk powder and tablets using RP-HPLC in 5.44 min.

The present work aimed at developing a new methodology using HPLC-UV-Vis to analyze a mixture of four drugs used in H. pylori treatment at isocratic mode and studying the effect of different parameters; mobile phase composition, buffer concentration and amount of additive on the analysis with the help of Design of Experiments that had been implemented.

2. Experimental

2.1 Chemicals and reagents

All chemicals and solvents used were of analytical HPLC grade. Standards solutions of omeprazole, metronidazole, clarithromycin, and amoxicillin were purchased from Sigma-Aldrich, USA. Methanol was provided from Lichrosolv, Germany, acetonitrile was obtained from Sigma-Aldrich Co., France. Regarding trimethylamine, it was provided from TEDIA- USA, where LC water and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Sigma-Aldrich - Germany. Sodium hydroxide was supplied from Sigma Aldrich - Sweden, potassium hydroxide was provided from Riedel-de Haen and finally, ortho-phosphoric acid was provided from Scharlau- China.

2.2 Instrumentations

HPLC (Dionex, Germany) with Chromeleon software (Version 6.8) was used in this study. HPLC is equipped with autosampler injector system, quaternary pump, UV-VIS detector and column oven. The analysis was performed on two RP C18 and C8 columns: Thermo Scientific Hypersil BDS C18 of (250 × 4.6 mm, 5µm) and Hypersil BDS C8 (150 × 4.6 mm, 5µm). The experimental design was carried out using Minitab, version 18. UV-Visible spectrometer (double beam- Cary E100) was used to get the maximum wavelength for the compounds.

2.3 Preparation of standard solutions

Stock solutions of omeprazole, metronidazole, clarithromycin and amoxicillin at 1000 ppm were prepared separately by dissolving 0.1g of each compound in: 100 mL methanol for metronidazole and clarithromycin, 100 mL water for amoxicillin, where omeprazole was dissolved in few drops 0.1 M NaOH then completed to 100 mL with methanol. The solution was then sonicated for 5 min. Regarding the standard mixture of the four drugs; the 100-ppm solution was prepared in pure water. Buffer solution of KH_2PO_4 was prepared in 500 mL at two

concentrations: 0.02 and 0.05 in water.

2.4 Design of Experiments (DOE)

Different HPLC conditions were evaluated on C18 column: composition of the mobile phase, buffer concentration and amount of additive added to the mobile phase. HPLC analysis was performed using isocratic elution with constant flow rate and injection volume during the whole experiments at 1.0 mL/min and 10 µL, respectively. Minitab software was used to create the DOE.

3. Results and Discussion

3.1 Detection of the wavelength maxima

The UV-Vis absorption spectrum of drugs was found in the region between 200– 800 nm as shown in Figure 3. The 205 nm has not been selected in this work since it showed interference with the mobile phases used in this study. Therefore, the suitable wavelength for the studied drugs was performed at 254 nm for amoxicillin and 304 nm for metronidazole, clarithromycin and omeprazole.

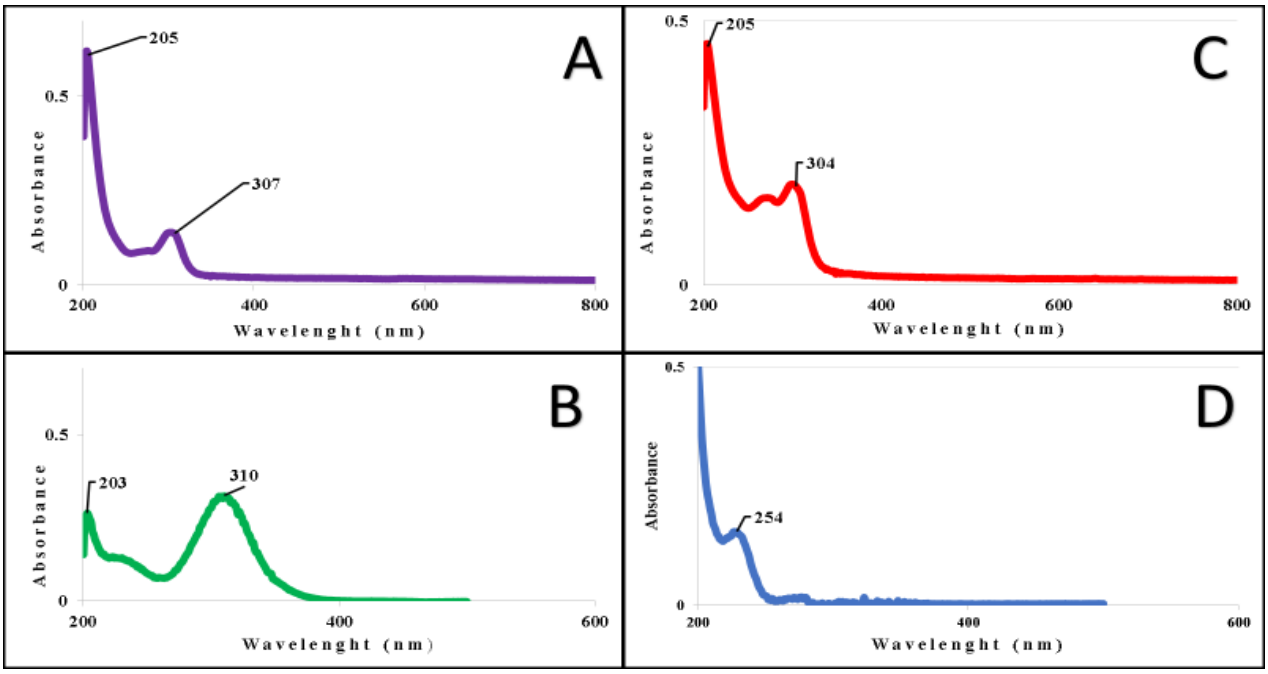

Figure 3. UV-Vis absorption spectra of (A) clarithromycin, (B) metronidazole, (C) omeprazole and (D) amoxicillin.

3.2 Selection of HPLC Column

As a preliminary test to choose the best stationary phase, a mixture of omeprazole, clarithromycin, amoxicillin and metronidazole was injected on HPLC. The results are presented in Figure 4.

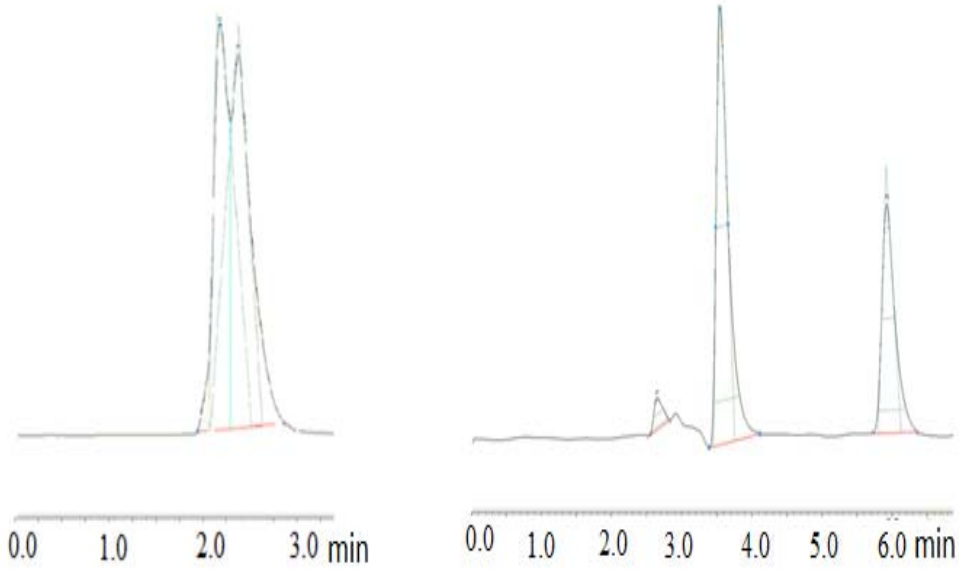

Figure 4. HPLC chromatograms of amoxicillin, clarithromycin, metronidazole and omeprazole at (33:17:50) ACN: MeOH: KH_2PO_4, respectively at 254 nm, flow rate 1.0 mL/min, injection volume 10 µL at room temperature. The tested columns are: A) C8 Hypersil BDS (150 x 4.6 mm. 5µ), and B) C18 Thermo scientific hypersil BDS (250 x 4.6 mm. 5µ).

The preliminary results showed that the C8 detected two peaks with retention time of less than 3.0 min, which was insufficient for the solutes to interact with the stationary phase and to separate them in proper resolutions. On the other hand, C18 column could detect three peaks with a retention time of around 6 min.

Hence, based on the preliminary test, Thermo Scientific Hypersil BDS C18 column was selected to continue the experimental work.

3.3 Effect of temperature

As known, increasing the temperature in HPLC analysis will decrease the viscosity, and then the retention time. Hence, in order to study the effect of mobile phase composition, concentration of buffer and the amount of additive on the analysis, the temperature should be determined and fixed. Herein, two temperatures: room temperature and 30°C have been tested on the chosen Thermo Scientific Hypersil BDS C18 column. The preliminary results showed that changing the temperature from room temperature to 30°C has very small effect on the separation asymmetric factor (As) and the plate height (H) (Table 2), it affects only the retention time as shown Figure 5 (A, B).

Therefore, we decided to implement the room temperature in the next steps.

Table 2. Separation results of the drugs mixture of amoxicillin and clarithromycin at different temperatures.

Condition	Amoxicillin			Clarithromycin		
	tr (min)	As	H	tr (min)	As	H
MeOH:KH_2PO_4 (25:75, v/v) 30°C	4.200	0.83	2.18	7.20	4.47	22.88
MeOH:KH_2PO_4 (25:75, v/v) at room temperature	4.600	0.86	2.03	7.50	3.96	22.77

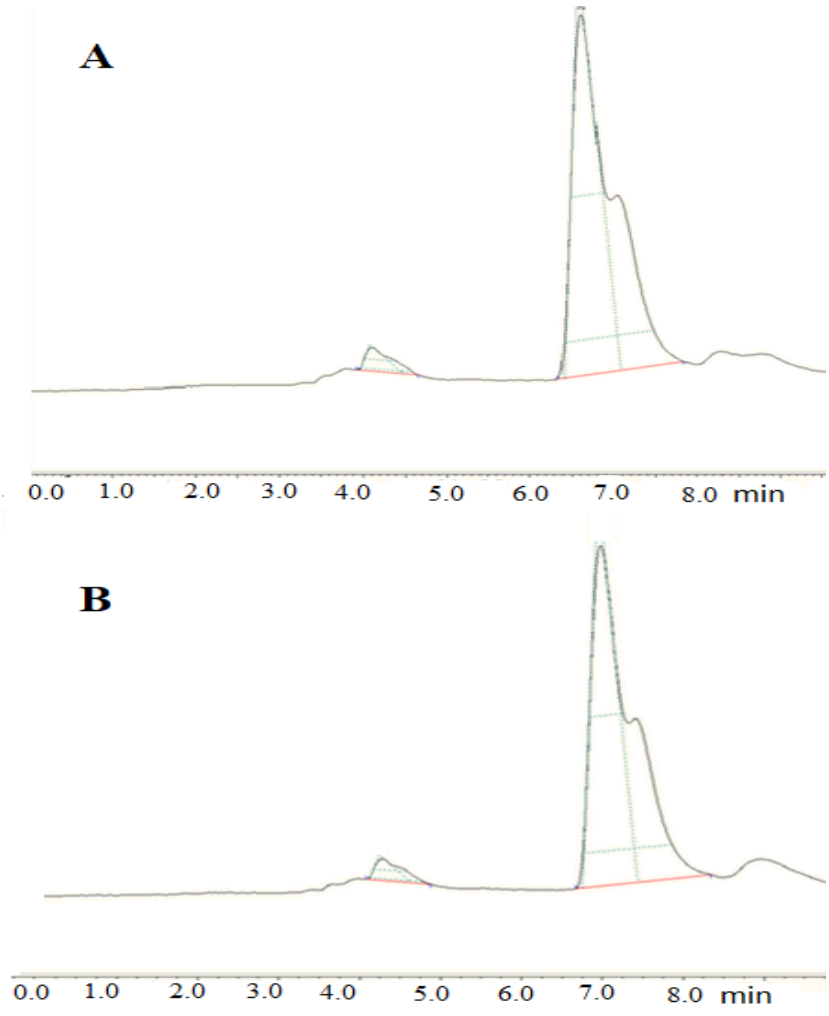

Figure 5. Chromatograms of amoxicillin and clarithromycin at pH 5.8, MeOH: buffer (25:75, v/v), flow rate 1.0 mL/min, injection volume 10 µL using C18 (250 × 4.6 mm, 5µm) column.

A) at 30°C and B) at room temperature.

3.4 Design of Experiments (DOE)

After choosing C18 Thermo Scientific Hypersil BDS column and room temperature as running parameters, Design of Experiments was built accordingly. The studied factors were: mobile phase composition, triethylamine

(TEA) amount and buffer concentration in order to choose the best parameters. For each condition, replicate samples have been injected and the average of results was registered. The results for these conditions were summarized in Table 3.

Table 3. DOE for the separation of amoxicillin, clarithromycin, metronidazole and omeprazole using C18 (250 x 4.6 mm, 5µ) column, flow rate of 1.0 mL/min at room temperature.

(ACN: MeOH: buffer) %	TEA (µL)	[buffer]	tr (min)
30:20:50	0	0.02	5.121 [A]* 6.408 [B & C]* 14.463 [D]*
30:20:50	0	0.05	2.559 [A] 3.211 [B & C] 7.019 [D]
30:20:50	30	0.05	5.097 [A] 6.361 [B & C] 13.656 [D]
30:20:50	30	0.02	2.553 [A] 3.165 [B & C] 6.334 [D]
20:20:60	0	0.02	2.649 [A] 3.604 [B & C] 15.842 [D]
20:20:60	0	0.05	2.658 [A] 3.706 [B & C] 18.992 [D]
20:20:60	30	0.02	2.419 [A] 2.662 [B & C] 17.478 [D]
20:20:60	30	0.05	2.668 [A] 3.628 [B & C] 15.772 [D]
20:30:50	30	0.02	3.417 [A] 4.473 [B, C & d]
20:30:50	30	0.05	2.597 [A] 3.244 [B & C] 7.875 [D]
20:30:50	0	0.05	2.724 [A] 3.358 [B & C] 9.657 [D]

(ACN: MeOH: buffer) %	TEA (µL)	[buffer]	tr (min)
20:30:50	0	0.02	2.638 [A] 3.322 [B & C] 9.564 [D]
30:30:40	0	0.02	4.966 [A] 6.068 [B & C] 9.955 [D]
30:30:40	0	0.05	2.653 [A] 3.054 [B & C] 5.275 [D]
30:30:40	30	0.02	2.502 [A] 3.039 [B & C] 5.122 [D]
30:30:40	30	0.05	2.562 [A] 3.036 [B & C] 4.839 [D]

*A: Amoxicillin, *B: clarithromycin, *C: metronidazole and *D: omeprazole.

Based on the experiments of the DOE, the best chromatogram observed was at (30: 30: 40) % ACN: MeOH: buffer, 30µL TEA, buffer concentration of 0.05 M (fixed at pH 6), since these conditions showed the best results in terms of reasonable retention time, less degradants peaks for amoxicillin and better peak shape compared to the other conditions (Figure 6).

Adding triethylamine (TEA; as ion pair reagent) to the mobile phase prevents tailing and band broadening of the peaks [48, 53]. In our results, when 30 µL TEA was added in comparison to 0 µL, the peak shape is enhanced, this is refers to the fact that TEA interacts strongly with silanols and inhibits them from interacting with amines in the drugs sample.

Regarding the buffer concentration, 0.05 M showed the best choice to improve the peak shape in comparison to 0.02M due to the increase of the ionic strength. Our result was in agreement with Darwish et al., [48] who studied the effect of buffer concentration (0.01, 0.03, 0.05 and 0.06 M). They found that 0.03 M was the best choice in comparison to 0.01 M since the peak shape enhanced due to ionic strength.

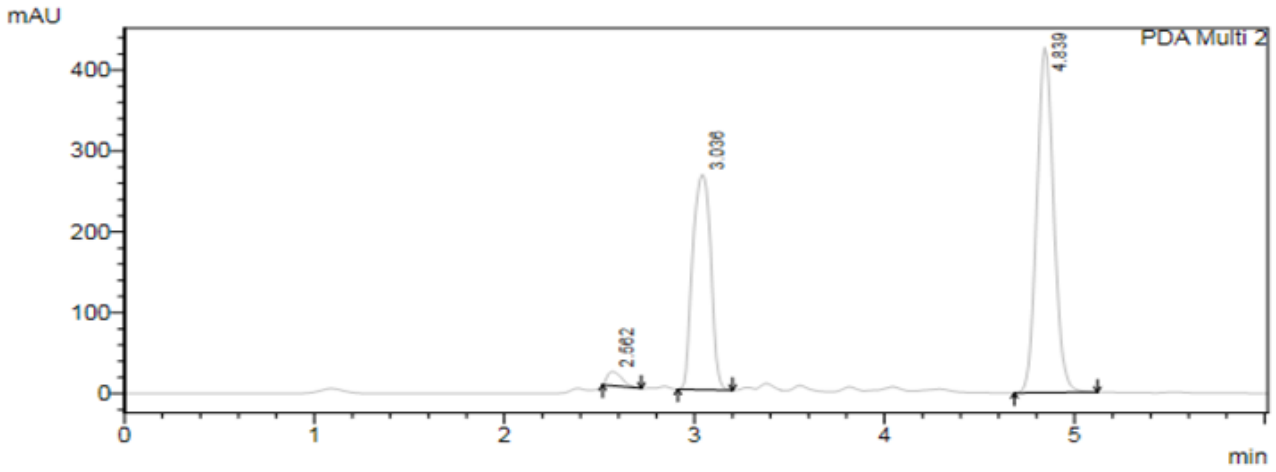

Figure 6. Chromatogram of amoxicillin (2.562 min), clarithromycin and metronidazole (3.036 min) and omeprazole (4.839 min) at (30: 30: 40)% of ACN: MeOH: buffer, respectively. A 30µL TEA at pH 6.0, flow rate of1.0 mL/min, injection volume 10 µL at room temperature using C18 (250 x 4.6 mm, 5µ) column at isocratic mode.

Conclusion

RP-HPLC method was developed in terms of mobile phase composition, buffer concentration and additive amount to separate the drugs mixture used as triple therapy for H. pylori treatment. A C18 reversed phase column showed better resolution than C8 for analyzing amoxicillin, metronidazole, omeprazole and clarithromycin at isocratic elution mode. The mobile phase compositions of 30:30:40 (ACN: MeOH: buffer) %, respectively showed the best choice in comparison to the other conditions. Regarding the optimum amount of trimethylamine added to the mobile phase to improve the separation, the outcomes showed that 30 µL was the best choice at pH around 6.0 with 0.05 M potassium dihydrogen phosphate as a buffer. The developed method could separate the mixture as following; amoxicillin and omeprazole at 2.56, 4.84, respectively, where metronidazole and clarithromycin were retained 3.04 min. Further investigations will be carried out in the future to separate metronidazole and clarithromycin.

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Conflict of interests

The authors declare that they have no conflict of interest

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تطوير طريقة جديدة باستخدام استِشراب السَّائِل الرَّفيع HPLC لتحليل الأدوية المستخدمة في علاج الجرثومة الملوية البوابية

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ملخص

الجرثومة الملوية البوابية (H-pylori) هي أكثر أنواع العدوى البكتيرية المزمنة التي تصيب الإنسان وتوجد في الطبقة المخاطية في المعدة وتتكيف مع الظروف الحمضية. بسبب مقاومة هذه الجرثومة، فإن العلاج الثلاثي مطلوب. في هذه الدراسة ، تم تطوير طريقة فصل باستخدام طريقة استشراب السائل الرفيع – الطور الثابت الغير قطبي، من حيث تكوين الطور المتحرك وتركيز مادة معادلة الحموضة والكمية المضافة لفصل خليط الأدوية المعقد المستخدم كعلاج ثلاثي لعلاج بكتيريا الملوية البوابية. أظهرت النتيجة الأولى أن عمود الطور الثابت الغير قطبي C18 له فاعلية فصل أفضل من C8 لتحليل : أموكسيسيلين وميترونيدازول وأوميبرازول وكلاريثروميسين. ثم تم تنفيذ تصميم التجارب لتقييم وتحديد أفضل ظروف الفصل. أظهرت النتائج أن الأموكسيسيلين تم فصله عند 254 نانومتر ، بينما تم الكشف عن ميترونيدازول وأوميبرازول وكلاريثروميسين عند 304 نانومتر. علاوة على ذلك ، تم تحقيق اعلى فاعلية فصل في تركيبات الطور المتحرك عند استخدام النسبة 30:30:40 (أسيتونيتريل: ميثانول: منظم الحموضة) ، على التوالي. فيما يتعلق بالكمية المثلى من ثلاثي ميثيل أمين المضاف إلى الطور المتحرك لتحسين فاعلية الفصل ، أظهرت النتائج أن 30 ميكرولتر كان الخيار الأفضل عند درجة الحموضة 6.0 مع 0.05 مول/لتر من فوسفات ثنائي هيدروجين البوتاسيوم كمنظم للحموضة. يمكن للطريقة المطورة فصل الخليط على النحو التالي؛ أموكسيسيلين وأوميبرازول عند 2.56 ، 4.84 دقيقة، على التوالي، بينما تم فصل الميترونيدازول والكلاريثروميسين عند الزمن 3.04 دقيقة.

الكلمات الدالة: استشراب السائل الرفيع بالطور الغير قطبي ، تصميم التجارب ، العلاج الثلاثي ، الحلزونية البوابية.

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Celecoxib inhibits cancer growth through cyclooxygenase 2 (COX2) independent pathways in HepG2 hepatocellular carcinoma

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ABSTRACT

Chronic inflammation has long been associated to carcinogenesis. Evidence has shown that nonsteroidal anti-inflammatory drugs (NSAIDs) may decrease the risk of certain types of cancer. In this study, we assessed the anti-proliferative effects of different NSAIDs against liver cancer using HepG2 cell line. In addition, we evaluated the combined effects of a selective COX2 inhibitor (Celecoxib) with chemotherapeutic drugs. The anti-proliferative and combined effects of COX2 inhibitors were evaluated by MTT assay. The effect of COX2 inhibitors on gene expression was assessed using real time PCR (RT-PCR). Also, the effect of COX2 inhibitors on colony formation was assessed through colony-formation assay. COX2 inhibitors treatment significantly inhibited the growth of HepG2 cells in a dose-dependent manner. The combined treatment of Celecoxib with either doxorubicin or raloxifene resulted in synergistic effects. In addition, Celecoxib treatment significantly reduced the expression of Bcl2 and VEGF genes in HepG2 cells, while the COX2 gene was not detected at all in HepG2 cell line suggesting that the anti-tumorigenic activity of Celecoxib is COX2 independent. Our results also revealed that COX2 inhibitors treatment significantly reduced the number and size of colonies formed by HepG2 cells.

Keywords: Hepatocellular Carcinoma, COX2 inhibitors, Celecoxib.

INTRODUCTION

Cancer is a general term that describes the state where cells at a certain part of the body grow and reproduce in an uncontrolled manner (1). Nowadays, cancer is considered one of the major causes of death around the world. It has moved from the third most common cause of death in 1990 to the second most common cause of death in 2013 next to heart disease (2).

Liver cancer or Hepatocellular Carcinoma (HCC) is one of the most frequent malignancies worldwide. Liver cancer is the second most frequent cause of death from cancer globally (3) and it's the sixth most commonly occurring cancer. Mainly, the risk factors for HCC are rolling around any agent leading to chronic hepatic injury, these factors can be summarized into the following: Hepatitis B virus (HBV) that accounts for 50% of all HCC cases and Hepatitis C virus (HCV) accounting for 25% of all cases of HCC (4). Although there are advancements in the treatments of HCC, its prognosis is still poor due to the recurrence and metastasis (5). While surgical resection and transplantation are the cornerstones of therapies in the early stage of the disease, regional therapy, and Sorafenib are beneficial in those with more advanced disease (6,7).

Cyclooxygenase 2 (COX2) is one of the molecular targets that have been increasingly investigated in many cancer types such as lung, colorectal, and breast cancer (8). It was shown that COX2 may have effects on many processes that are involved in different stages of carcinogenesis including angiogenesis, apoptosis, immune

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function, tumor growth, and invasiveness (8). Several studies illustrate the strong relationship between COX2 enzyme overexpression and Hepatocellular Carcinoma progression and grading and may give an idea about the role of this protein in hepatocarcinogenesis (9).

NSAIDs are of the most used groups of drugs worldwide to treat inflammation and pain. However numerous side effects are reported due to the nonselective action on both COX1 and COX2 enzymes (10). The more selective drugs that are called Coxibs or selective COX2 inhibitors have higher selectivity toward COX2 enzymes and low affinity toward COX1 (11). It has been demonstrated that COX2 inhibitors suppressed the growth of the HCC cell lines in a time and dose-dependent manner especially for cells expressing COX2 protein (12).

In this study, we aimed to investigate the expression pattern of COX2 protein in Hepatocellular Carcinoma, especially the HepG2 cell line, and to determine its clinical significance. Besides, the anti-tumorigenic effects of selective COX2 inhibitors against Hepatocellular Carcinoma were evaluated.

Materials and Methods

Human Hepatocellular Carcinoma cell line HepG2 and human Fibroblast cells were purchased from American Type Culture Collection (ATCC, USA), Celecoxib, Etoricoxib, Indomethacin, Aspirin and Raloxifene hydrochloride were all from (Sigma-Aldrich, USA), Doxorubicin hydrochloride 2mg/ml (Ebewe Untrerach, Austria), Direct-zol to RNA (miniprep KIT, USA), Primescript to Master mix KIT (Takara, Japan), PCR TB Green Premix Ex Tag to 2 (Tli RNase PLUS) KIT (Takara, Japan).

HepG2 cell line was cultured in DMEM culture medium supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, 1% non-essential amino acid, and 1% sodium pyruvate. Cells were incubated in 5% CO_2, 95% humidified air at 37°C.

Cell Viability Assay

Anti-proliferative activity of drugs on the HepG2 cell line was assessed using MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay as previously described (13). HepG2 cells were plated in 96-well plates at concentration 8000 cells per 100 µl in DMEM medium and incubate at 37°C, after 24 h the media was discarded and the cells were treated with drugs with increasing doses of 50-350 µM for Etoricoxib, Indomethacin, and Aspirin, and 30-160 µM for Celecoxib conditions. After treatment addition for 24 h, 48 h, and 72 h incubation periods 10 µl MTT dye at concentration 5 mg/ml was added for each well. The optical density was measured at 570 nm and 630 nm wavelength using a microplate reader (µ Quant Plate Reader, Biotek, USA).

Drug Combination with Doxorubicin and Raloxifene

Hepatocellular Carcinoma cell line HepG2 was seeded in 96-well plates (2 replicate/group) for 24 h, to observe the combined outcome of Celecoxib on Doxorubicin and Raloxifene. Cells were treated with a range of concentrations of Celecoxib, Doxorubicin, and Raloxifene alone and in combination. The combination ratios of doxorubicin with Celecoxib were (1:5,1:20 and 1:50), the combination ratios of Raloxifene and Celecoxib were (1:5, 1:10, and 1:20). After 24, 48, and 72 hours of treatment MTT dye was added, and viability was measured. The Combination Index (CI) was calculated using Compusyn software based on the Chou-Talalys combination index theorem.

$$CI = \frac{D1}{DX1} + \frac{D2}{DX2}$$

Where (Dx)1 =dose of drug 1 to produce 50% cell kill alone, (D)1 = dose of drug 1 to produce 50% cell kill in combination with (D)2. (Dx)2 = dose of drug 2 to produce 50% cell kill alone, (D)2 = dose of drug 2 to produce 50% cell kill in combination with (D)1. The Combination Index CI values for combined treatment are shown in Table 2. Overall CI < 1 indicates a synergistic effect, while CI=1 signifies additive effect and CI >1 means antagonistic effect.

Colony Formation Assay

HepG2 cells were seeded in 6 well plates and could attach over the night. After 24 h of seeding each well was treated with one of the following: Celecoxib IC_{50}, Celecoxib half IC_{50}, Etoricoxib IC_{50}, Etoricoxib half IC_{50}, and untreated control wells containing only media. After 48 h of treatment, the media was discarded, the cells were detached by trypsin and counted using a hemocytometer. To prepare for the assay, two layers of agar were prepared, the first layer consisted of equal volumes of 2X media and 1% agar. After preparation of this mixture 2 mL of it was added to each well and allowed to solidify. The upper layer consisting of 1mL of 0.6% noble agar and a total of 1 ml of medium containing cell pellets that were previously collected and left to solidify. After that, the plate was incubated at 37°C in a humidified incubator for 2-3 weeks and was monitored every other day to see the colonies' growth, photos were taken after 20 days of incubation using an Evos microscope.

Extraction of Cellular RNA and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA extraction was carried out according to Direct-Zol TM RNA Miniprep kit instructions using Triazol reagent. Subsequently, cDNA was synthesized using Primescript TM RT Master Mix kit instructions to produce the cDNA. Reverses Transcriptase - Polymerase Chain Reaction (RT-PCR) was performed using TB Green Premix kit using 4 primers *Bcl-2*, *VEGF*, *COX2* and *GAPDH* with its forward and reverse sequences, as previously described (14).

RESLTS

Effect of COX Inhibitors on the Viability of Hepatocellular Carcinoma Cell line HepG2.

To examine the effects of COX inhibitors (Celecoxib, Etoricoxib, Indomethacin, and Aspirin) on the viability of HepG2 cell line, MTT assay was carried out, and the results are shown in Figure 1. All drugs were tested in three independent experiments after 24 h, 48 h, and 72 h incubation periods. According to the results, Celecoxib significantly inhibited the growth of cells compared to the untreated control cells in a dose and time-dependent manner. Other COX inhibitors also yielded inhibition in growth but with higher 50% inhibitory concentrations (IC_{50}). The anti-proliferative effect of other COX inhibitors significantly varied. Etoricoxib has IC_{50} of 260.9 µM after 48 h, and 310.1 µM after 72 h incubation time. Aspirin always has the highest IC_{50} values among the four examined drugs indicating the worst activity.

Effect of Chemotherapeutic Agents on the Viability of HepG2 Cell Line

The effect of known chemotherapeutic agents such as Doxorubicin and Raloxifene on the viability of HepG2 cell line after 24 h, 48 h, and 72 h is shown in Figure 2. Treatment of HepG2 cell line with Doxorubicin in a concentration range of 1.5-10 µM obviously inhibited the cell viability in a time and dose-dependent manner. This result was also observed upon the treatment of HepG2 cells with Raloxifene in a range of 12.5-200 µM. Such treatment yielded growth inhibition in a time and dose-dependent manner. The 50% inhibitory concentration (IC_{50}) values for the examined chemotherapeutic agents Doxorubicin and Raloxifene against HepG2 cell line are shown in Table

Effect of Combined treatment of Celecoxib and Chemotherapeutic Agents on HepG2 Cell Line

This study evaluated the combined effect of COX2 selective inhibitor Celecoxib and standard chemotherapeutic agents of Doxorubicin or Raloxifene. Cells were treated with Celecoxib and Doxorubicin at combination ratios of 1:5,1:20, and 1:50. The results revealed that treatment with Celecoxib and Doxorubicin in all combination ratios significantly decreased cancer cell viability compared to treatment with Doxorubicin alone, as shown in Figure 3, and this combination reduced the IC_{50} of Doxorubicin on HepG2 cells by 2 folds. Besides, treatment of HepG2 cells with Raloxifene and Celecoxib at combination ratios of 1:5, 1:10, and 1:20 reduced cell

viability when compared to Raloxifene treatment alone in the first 24 h, as shown in Figure 3, and this decreased the IC_{50} of Raloxifene on HepG2 cells by almost 4 folds.

Effect of COX2 Selective Inhibitor Celecoxib on Genes Expression.

The effects obtained from the treatment of HepG2 cells with selective COX2 inhibitor Celecoxib on gene expression were investigated with RT-PCR experiment upon the use of four genes; *GABDH* as a housekeeping gene, and three examined genes including *Bcl2*, *VEGF*, and *COX2*. The results showed that untreated HepG2 cells express the *Bcl2* gene, an anti-apoptotic gene, at a level that is 75% higher than the level of expression in the control fibroblast cells, a model for normal cells. Upon the treatment of HepG2 cells with 0.1 IC_{50} and 0.25 IC_{50} of Celecoxib, the relative gene expression of *Bcl2* was changed according to the concentration of drug used. Interestingly, 0.1 IC_{50} resulted in a 25% reduction in Bcl2 gene expression relative to the untreated HepG2 cells. On the other hand, treatment with 0.25 IC_{50} yielded a 40% reduction in *Bcl2* gene expression relative to the untreated HepG2 cells, as shown in Figure 4 (A). Also, the basal level of *VEGF* gene expression, an important gene for angiogenesis and vascularization of tumor cells, was pronounced in HepG2 untreated cells with more than 25% relative to the basal gene expression in fibroblast cells. When treated with 0.1 IC_{50} and 0.25 IC_{50} of Celecoxib, the reduction in gene expression was 55% and 50% respectively Figure 4 (B). Remarkably, the *COX2* gene was not detected at all in HepG2 cells while it was detected in Fibroblast cells. This result may indicate that different pathways that are COX2 independent may be involved in the observed anti-proliferative activities of Celecoxib.

Effect of COX Inhibitors on Colony Formation in HepG2 cells

Colony Formation Assay(CFA) is an in vitro cell survival assay that depends on the ability of separate cells to grow up into colonies (14). The results demonstrated that cells treated with IC_{50} of Celecoxib yielded fewer colonies compared to the control untreated HepG2 cells, and these colonies were smaller in size than the colonies in the control well. Regarding cells treated with half IC_{50} of Celecoxib, although a lower number of colonies was noticed when compared with the control wells, the effect on size was less obvious than the effect of a higher concentration of Celecoxib, as shown in Figure 5(A). Concerning Etoricoxib, its IC_{50} concentration affected the number of colonies significantly with fewer colonies compared to the control group, but its effect on size was less pronounced than the effect of Celecoxib on size. Half IC_{50} of Etoricoxib produced less effect than its IC_{50} with a lower number of colonies than the control but more than the of IC_{50} Etoricoxib as shown in Figure 5(B).

DISCUSSION

Several studies illustrated the strong relationship between COX2 enzyme overexpression and hepatocarcinogenesis, indicating the role of this enzyme in Hepatocellular Carcinoma progression and grading (9). According to the available data, the regulation of the COX2 signaling pathway was a vital source to try new drugs and new therapeutic approaches on HCC (15).

The present study has demonstrated that selective COX2 inhibitors Celecoxib significantly inhibited the growth of HepG2 cells compared to untreated control cells, with IC_{50} of around 55 µM and this finding is consistent with other studies (8), (12). COX inhibitors induced cell death in a concentration-dependent manner in HepG2 cells. In addition, compared to literature, it was reported that COX inhibitors, specifically COX2 selective inhibitors exerted their anti-proliferative effect on various cancer types like esophageal squamous cell carcinoma (16), and Familial adenomatous polyps (17).

In this study, Celecoxib was the most active drug among the tested drugs, while Aspirin was the least potent one. Such a result can be attributed to the fact that Celecoxib may work through different mechanisms of action on cancer cells and not only targeting COX2

enzymes, such mechanisms may include modulating of gene expression as was reported in different studies (18). Dai *et al*. reported that Celecoxib may exert its anti-tumor activity by affecting PNO1 expression and interfering with the AKT/mTOR signaling pathway in hepatocellular carcinoma (HCC) (18).

Traditional methods of cancer treatment have limited success due to systemic side effects, development of drug resistance, and sub-optimal drug concentration at the tumor site (19). Multi-target inhibitors or formulations are an attractive alternative to traditional therapy which synergistically inhibit multiple pathways that are essential for the growth of cancer cells. This study demonstrated the beneficial combined effects of Celecoxib with Doxorubicin and Raloxifene. Doxorubicin is a type of chemotherapeutic agents called anthracyclines. It slows or stops the growth of cancer cells by blocking an enzyme called Topoisomerase 2, Doxorubicin is given intravenously and used to treat multiple cancers like Breast, lung, and multiple myeloma (20). However Multiple cytotoxic effects on multiple organs and tumor resistance limit its clinical uses (21). Besides, Raloxifene is a selective estrogen enzyme modulator that induces an antagonistic effect on estrogen enzyme in breast tissues (22), Raloxifene has been approved by FDA for prevention and treatment of invasive estrogen enzyme-positive breast cancer in postmenopausal women, but it also increases the risk for venous thromboembolism (23). According to these results, the beneficial effect of combined treatment may be due to enhance the anti-proliferative effect of the chemotherapeutic agent, reduce the resistance, and decrease side effects by using a less concentration of such drugs while maintaining the same effectiveness.

The synergistic effect between Doxorubicin and Celecoxib has been reported before in skin cancer, and the researchers believed that this effect was due to the inhibition of multiple key signaling pathways like protein kinase B (AKT) which is a mediator of signal transduction that has a central role in tumorigenesis and cancer development and COX2 expression (24). The mechanism of synergistic inhibition of these drugs on Hepatocellular Carcinoma is not completely clear and needs further investigation. Regarding Celecoxib and Raloxifene, a synergistic inhibition effect has been detected after 24 h of treatment. Raloxifene alone may produce an apoptotic effect on HepG2 cell through activation of the Aryl hydrocarbon enzyme (AhR) signaling pathway that leads to apoptosis in HepG2 cells according to O'Donnell *et al.* (2014). The synergistic inhibition between Celecoxib and Raloxifene on Hepatocellular Carcinoma has not been studied before, so our study is the first report validating this unique combination.

P- glycoprotein is overexpressed in many cancer cells, it could extrude excessive effect chemotherapeutic drugs from cells and prevent the cytotoxic effect. It was reported that Celecoxib reduced p-glycoprotein expression and this produced an apoptotic effect on cells (26), also some studies stated that Raloxifene increases the P-glycoprotein ATPase activity and accordingly it will decrease the p-glycoprotein activity (27), so the synergistic effect between Celecoxib and Raloxifene may be through the activity on p- glycoprotein.

The mechanism of anti-cancer activity of Celecoxib has been described via COX-dependent and independent pathways (28),(29). In this study, the effect of Celecoxib on gene expression on three genes was studied *Bcl2*, *VEGF-A*, *COX2*, and *GABDH* as a housekeeping gene.

Bcl2 was the first anti-death gene discovered. Bcl2-family protein regulates all types of cell death including apoptosis, necrosis, and autophagy. Overexpression of this gene is related to anti-apoptotic proteins and demonstrated to inhibit cell death. According to our results, it can be concluded that Celecoxib induced apoptosis to HepG2 through the reduction of Bcl*2* gene expression**.** The effect of Celecoxib on modulating *Bcl2* gene expression has been reported before (30) (31), and other data demonstrated that Celecoxib induced apoptosis through

reducing the level of the anti-apoptotic protein Bcl2 in H22 hepatoma cells (32), however, this is the first time to report such finding in HepG2 cell line.

Concerning the *VEGF-A* gene, it is the most prominent and well-researched regulator of angiogenesis in cancer cells (33). It was reported that Celecoxib inhibits vascular endothelial growth factor expression and reduces angiogenesis in human pancreatic cancer (34). The reduction of *VEGF* gene expression in Hepatocellular Carcinoma's HepG2 cell line is studied for the first time here.

Results regarding Cyclooxygenase 2 (*COX2)* gene expression were interesting, COX2 was not detected at all in HepG2 cells, but it was detected in Fibroblast cells. Our results are consistent with previously reported data, which concluded that HepG2 cell line does not contain *COX2* genes(35). Accordingly, it can be concluded that the effect of Celecoxib on HepG2 cells is independent of the COX2 enzyme.

Clonogenic assay or colony-forming assay enables an assessment of the difference in proliferative capacity between control untreated cells and cells that have undergone various treatments, to provide a further measure of cell death (36-38). This assay can yield important information about the long-term proliferative potential of cells that cannot be determined by short-term assays.

In this study, HepG2 was divided into groups, and each group was subjected to different treatment, IC_{50} of Celecoxib, half IC_{50} of Celecoxib, IC_{50} of Etoricoxib, and half IC_{50} of Etoricoxib and control group. Celecoxib IC_{50} decreases the number and size of colonies formed by HepG2 cells significantly. Half IC_{50} of Celecoxib decrease the number of colonies but its effect on size was less obvious than IC_{50} of Celecoxib, all these effects were compared to untreated HepG2 cells. Regarding Etoricoxib, its IC_{50} effect on colony formation in HepG2 cells was pronounced, it decreased the number and size of colonies formed by HepG2 compared to untreated cells, half IC_{50} of Etoricoxib also produced colonies with less size and number than control but higher than IC_{50} well.

CONCLUSION

COX inhibitors (Celecoxib, Etoricoxib, Indomethacin, and Aspirin) inhibited the growth of HepG2 cells in a concentration and time-dependent manner. Celecoxib was the most powerful agent and Aspirin was the least potent. Celecoxib anti-proliferative activity is mediated through the induction of apoptosis by downregulation of the Bcl2 gene. Combination of Celecoxib with Doxorubicin resulted in synergistic inhibition of HepG2 cell viability at all incubation periods 24 h,48 h, and 72 h, while the combination with Raloxifene resulted in a synergistic effect after 24 h. COX inhibitors, Celecoxib and Etoricoxib, significantly lower the size and number of colonies formed by HepG2 cells. Celecoxib modulated gene expression in HepG2 cell line, it decreased the expression of Bcl*2* and *VEGF-A* genes. Most interestingly, the COX2 gene wasn't detected in HepG2 cell line suggesting a COX2 independent pathway that is involved in the observed COX2 inhibitor anti-proliferative activity.

Figure 1A

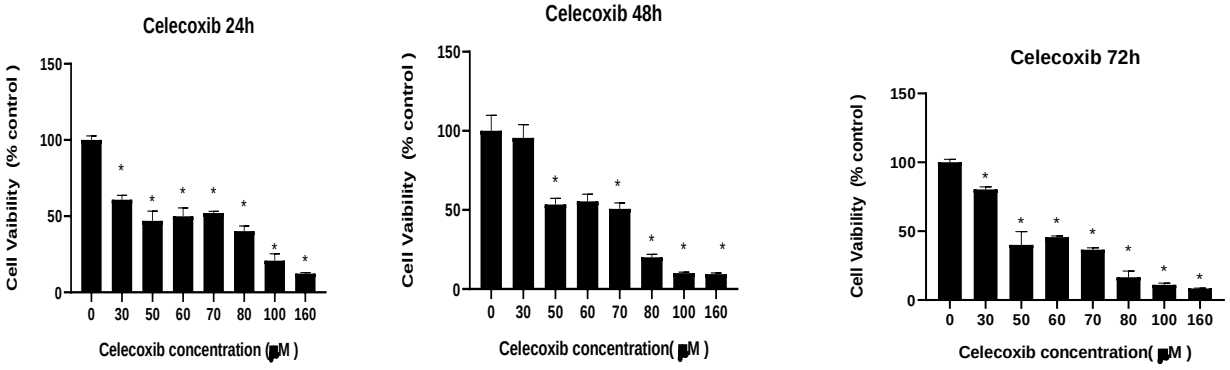


Figure 1: Effect of COX inhibitors on HepG2 cells viability.

(A) Effect of Celecoxib on the viability of HepG2 cell line. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate in three independent experiments. ***** P< 0.05 is significantly different from the untreated control group.

Figure 1B:

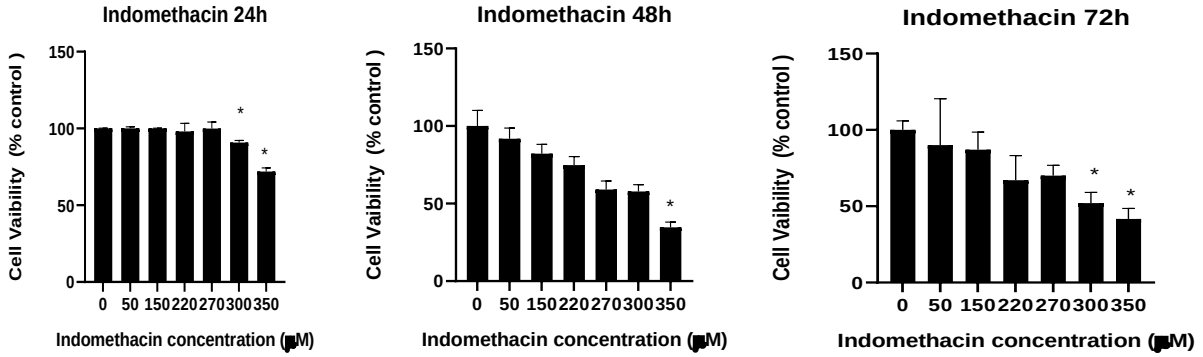


Figure 1: (continued) Effect of COX inhibitors on HepG2 cells viability.

(B) Effect of Indomethacin on the viability of HepG2 cell line. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate in three independent experiments. ***** P< 0.05 is significantly different from the untreated control group.

Figure 1C:

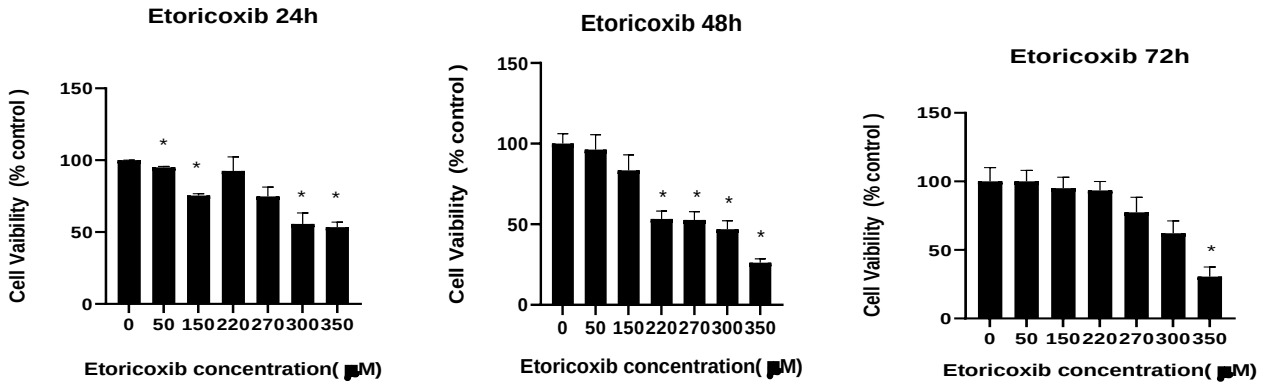


Figure 1: (continued) Effect of COX inhibitors on HepG2 cells viability.

(C) Effect of Etoricoxib on the viability of HepG2 cell line. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate in three independent experiments. ***** P< 0.05 is significantly different from the untreated control group.

Figure 1D:

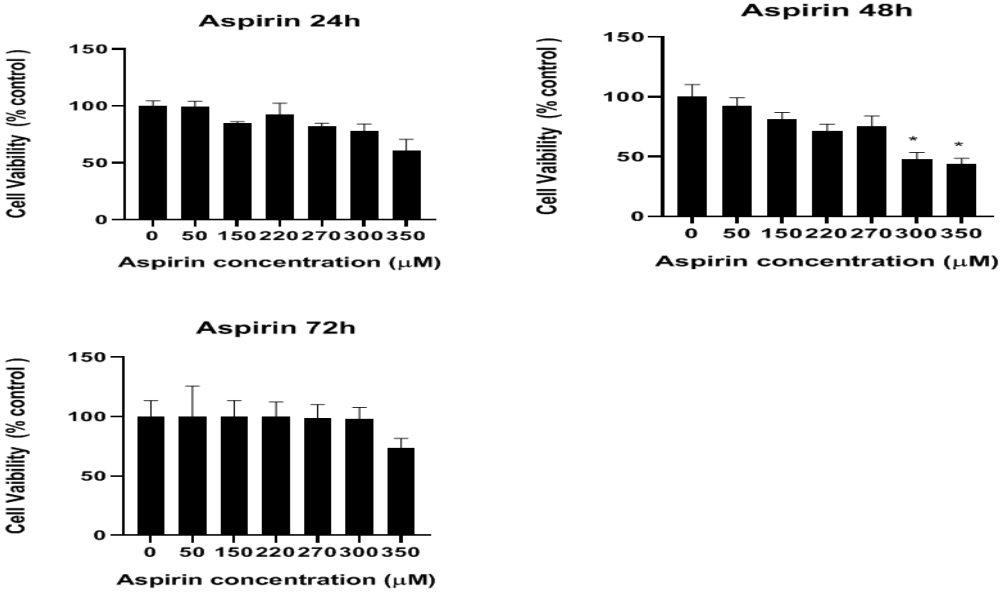


Figure 1: (continued) Effect of COX inhibitors on HepG2 cells viability.

(D) Effect of Aspirin on the viability of HepG2 cell line. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate in three independent experiments. ***** P< 0.05 is significantly different from the untreated control group.

Figure 2A:

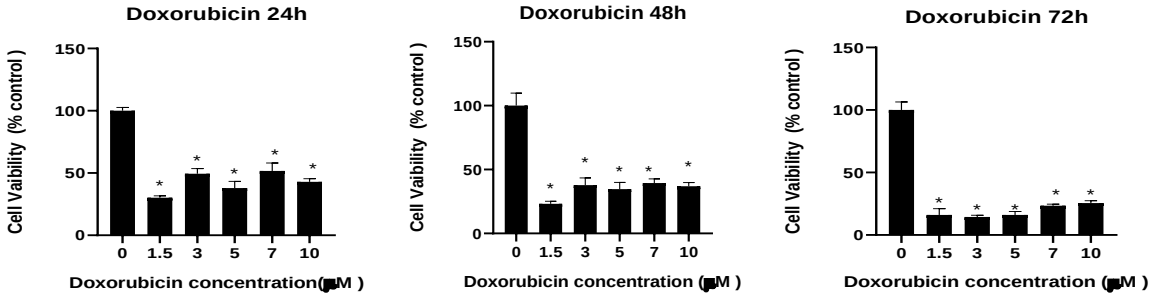


Figure 2: Effect of chemotherapeutic agents on HepG2 cells viability.

(A) Effect of Doxorubicin on the viability of HepG2 cell line. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate in three independent experiments. ***** P< 0.05 is significantly different from the untreated control group.

Figure 2B:

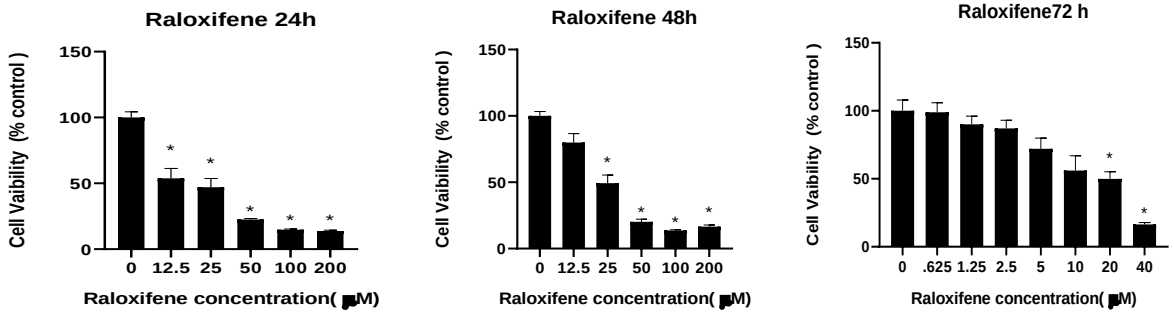


Figure 2:(continued) Effect of chemotherapeutic agents on HepG2 cells viability.

(B) Effect of Raloxifene on the viability of HepG2 cell line. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate in three independent experiments. ***** P< 0.05 is significantly different from the untreated control group.

Table 1: The 50% inhibitory concentration (IC_{50}, µM) ± SD for COX inhibitors, Doxorubicin, and Raloxifene against HepG2 cell line at different incubation periods

Treatment	24 h	48 h	72 h
Celecoxib	54±1.28	60±1.9	50±2.0
Indomethacin	378.5±3.8	308.3±6.0	349.1±4.0
Etoricoxib	398.5±8.5	260.9±7.8	310.1±7.6
Aspirin	444.4±8.0	334.5±10.0	363.6±5.6
Doxorubicin	3.9±0.1	2.4±0.08	1.45±0.05
Raloxifene	17.8±1.0	25.7±1.3	14.0±0.5

Figure 3A

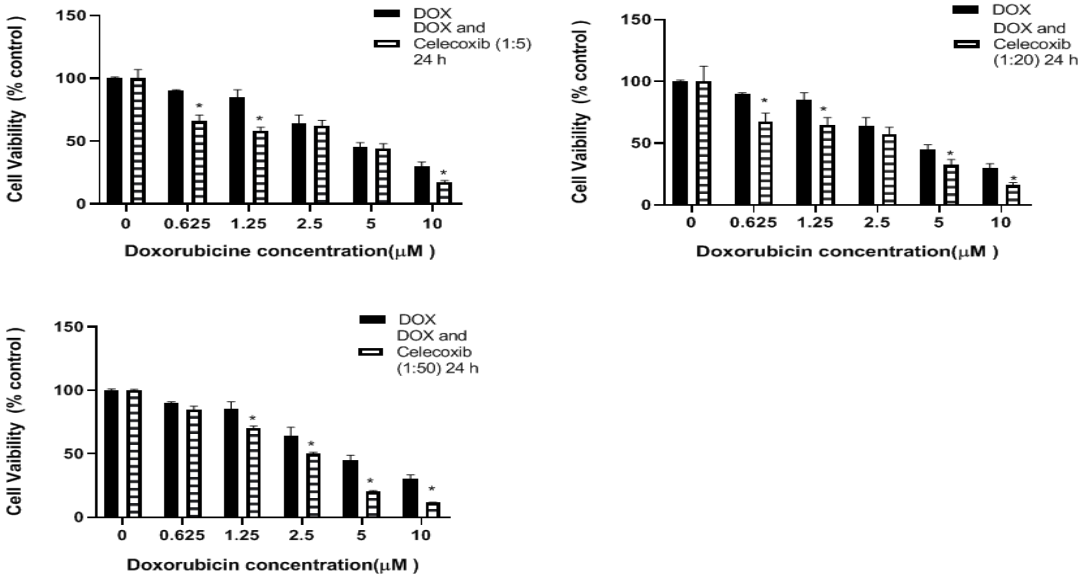


Figure 3: Effect of combination of Celecoxib and chemotherapeutic agents on the viability of HepG2 cell line. **(A)** Effect of combination between Celecoxib and Doxorubicin on HepG2 cells for 24h. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate repeated three independent times. *P< 0.05 is significantly different from the respective chemotherapeutic treatment alone.

Figure 3B

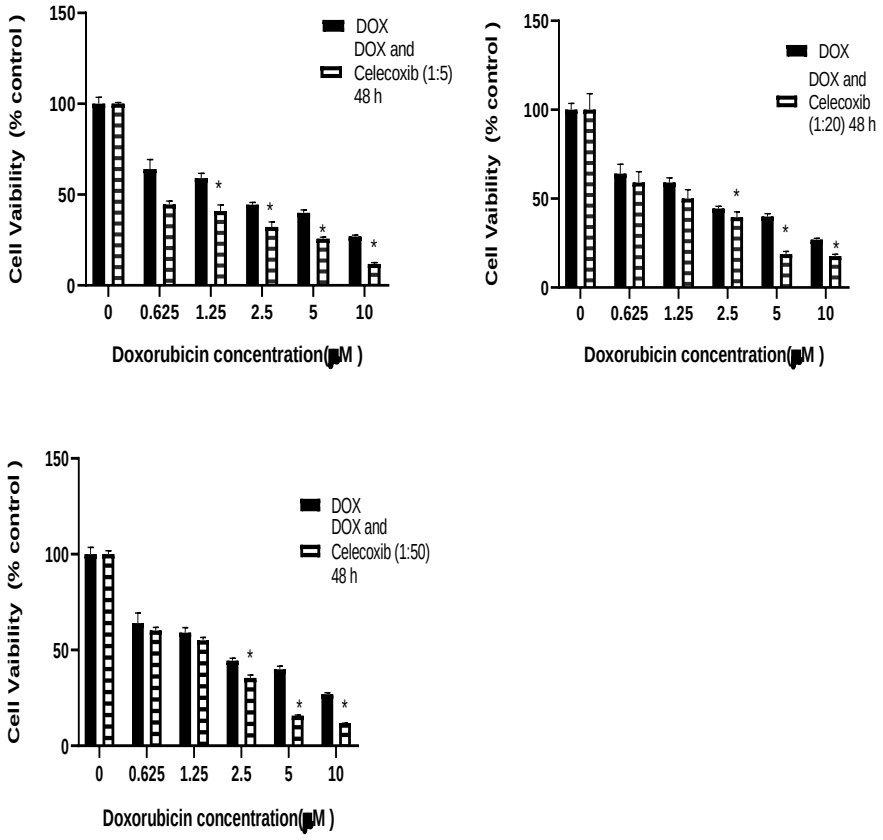


Figure 3:(continued) Effect of combination of Celecoxib and chemotherapeutic agents on the viability of HepG2 cell line. (B) Effect of combination between Celecoxib and Doxorubicin on HepG2 cells for 48h. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate repeated three independent times. *P< 0.05 is significantly different from the respective chemotherapeutic treatment alone.

Figure 3C

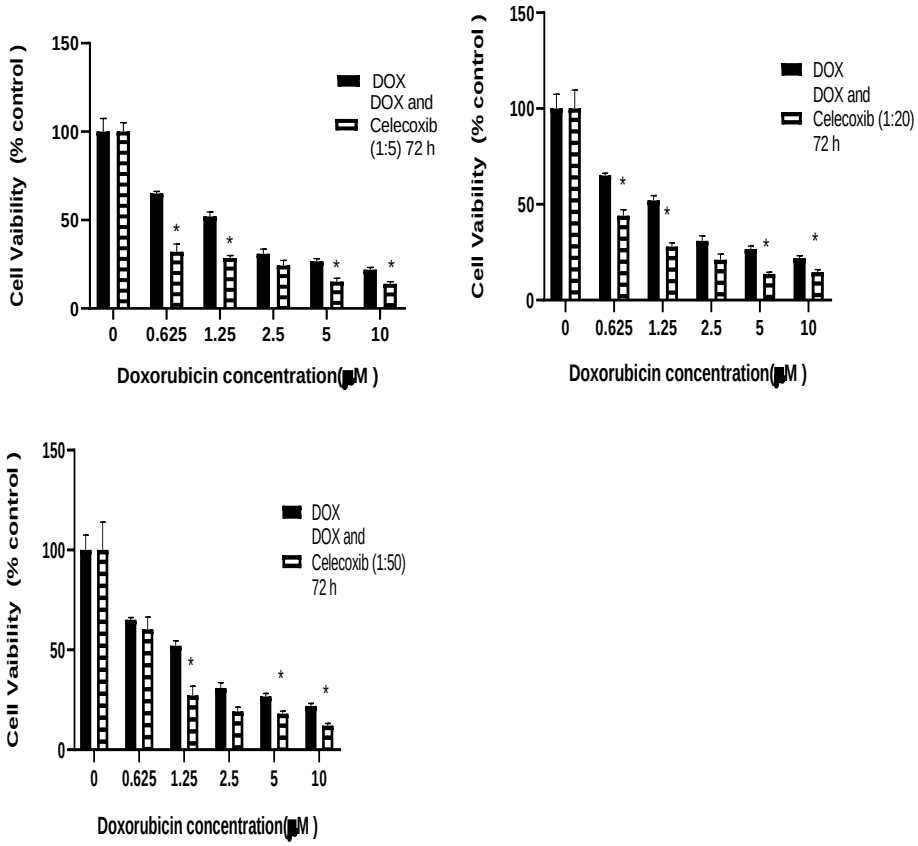


Figure 3:(continued) Effect of combination of Celecoxib and chemotherapeutic agents on the viability of HepG2 cell line. (C) Effect of combination between Celecoxib and Doxorubicin on HepG2 cells for 72h. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate repeated three independent times. *P< 0.05 is significantly different from the respective chemotherapeutic treatment alone.

Figure 3D

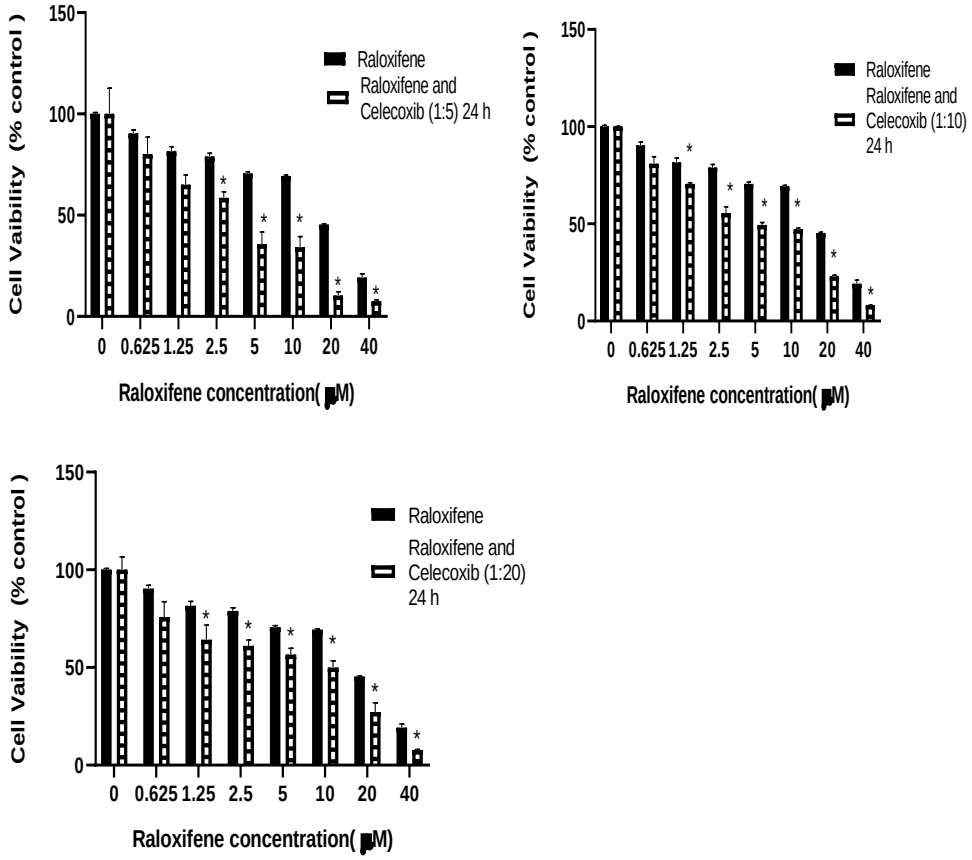


Figure 3:(continued) Effect of combination of Celecoxib and chemotherapeutic agents on the viability of HepG2 cell line. (D) Effect of combination between Celecoxib and Raloxifene on HepG2 cells for 24h. The data shown represent the mean percentages of cell viability ± SD. Each experiment was performed in duplicate repeated three independent times. *P< 0.05 is significantly different from the respective chemotherapeutic treatment alone.

Table 2: Combination Index (CI) values for combined treatment of Celecoxib and chemotherapeutic agents against HepG2 cell line.

Combination	Combination Ratio	CI 24 h	CI 48 h	CI 72 h
Doxorubicin+ Celecoxib	1:5	0.6	0.8	0.3
Doxorubicin+ Celecoxib	1:20	0.4	0.6	0.2
Doxorubicin+ Celecoxib	1:50	0.2	0.45	0.5
Raloxifene+ Celecoxib	1:5	0.3	NA	NA
Raloxifene+ Celecoxib	1:10	0.5	NA	NA
Raloxifene+ Celecoxib	1:20	0.4	NA	NA

Figure 4A:

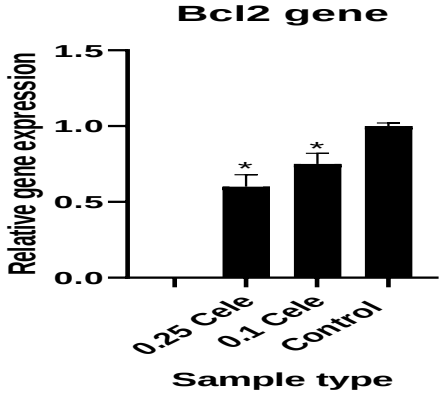


Figure 4: Change in relative gene expression with different sample types.

(A) Effect of Celecoxib on *Bcl2* gene expression in HepG2 cells. 0.25 Cele represents treatment with 0.25 IC_{50} of Celecoxib, 0.1 Cele represents treatment with 0.1 IC_{50} of Celecoxib, control represents control untreated HepG2 cells. The data shown represent the mean percentages of relative gene expression ± standard deviation (SD). Each experiment was performed in duplicate. *P< 0.05 significantly different from respective control HepG2 cells.

Figure 4B

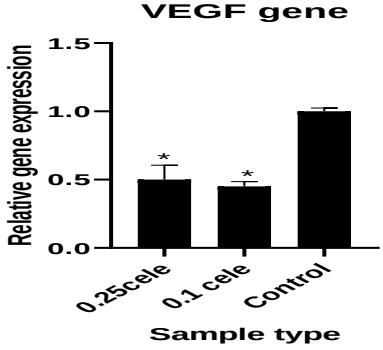
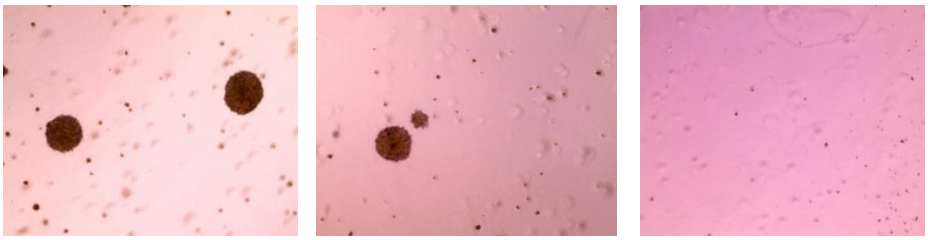


Figure 4:(continued) Change in relative gene expression with different sample types.

(B) Effect of Celecoxib on *VEGF* gene expression in HepG2 cells. 0.25 Cele represents treatment with 0.25 IC_{50} of Celecoxib, 0.1 Cele represents treatment with 0.1 IC_{50} of Celecoxib, control represents control untreated HepG2 cells. The data shown represent the mean percentages of relative gene expression ±SD. Each experiment was performed in duplicate. *P< 0.05 significantly different from respective control HepG2 cells.

Figure 5A:

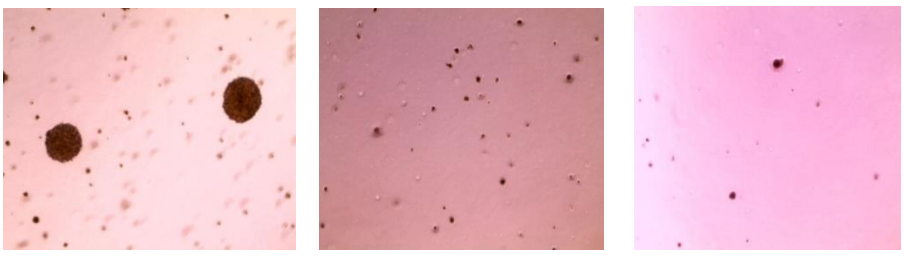


Control group **Half IC_{50} of Celecoxib** **IC_{50} of Celecoxib**

Figure 5: The effect of COX inhibitors on colony formation.

(A) Colony formation assay was performed on HepG2 cells, with control non treated group, group treated with IC_{50} of Celecoxib, and other treated with half IC_{50} of Celecoxib.

Figure 5B:



Control group **Half IC_{50} of Etoricoxib** **IC_{50} of Etoricoxib**

Figure 5: (continued) The effect of COX inhibitors on colony formation

(B) Colony formation assay was performed on HepG2 cells, with control non treated group, group treated with IC_{50} Etoricoxib, and other treated with half IC_{50} Etoricoxib.

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التحقيق في الآليات الجزيئية الكامنة وراء النشاط المضاد للتكاثر والمضاد للورم لمثبطات انزيم COX2 ضد خلايا سرطان الكبد

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ملخص

يعد سرطان الكبد أو سرطان الخلايا الكبدية أحد أكثر أنواع الأورام الخبيثة شيوعًا في جميع أنحاء العالم. على الرغم من وجود تطورات في علاج سرطان الخلايا الكبدية، إلا أن تشخيصه لا يزال ضعيفًا بسبب تكرار حدوثه وانتشاره. في هذه الدراسة ، لقد تم تقيم التأثيرات المضادة للتكاثر والمضادة للهجرة لمثبطات COX2 في خلاياHepG2 ، وهو أحد انواع خلايا سرطان الكبد. بالإضافة إلى ذلك ، قمنا بتقييم التأثيرات المشتركة لمثبطات COX2 الانتقائية (سيليكوكسيب) مع أدوية العلاج الكيميائي ، وكذلك الآلية التي تقوم بها هذه المثبطات لقتل الخلايا السرطانية. تم تقييم التأثيرات المضادة للتكاثر والتأثيرات المشتركة لمثبطات COX2 مع ادوية العلاج الكيميائي بواسطة فحص ال .(MTT)تم تقييم تأثير مثبطات COX2 على التعبير الجيني باستخدام .RT-PCR وتم تقييم اثرها على تكوين المستعمرات باستخدام فحص تشكيل المستعمرات . لقد أظهرت النتائج أن مثبطات COX2 تعمل بشكل كبير على تثبيط نمو خلايا سرطان الكبد HepG2 بطريقة تعتمد على التركيز. نتج عن العلاج المشترك لسيليكوكسيب مع دوكسوروبيسين و رالوكسيفين تأثيرًا تآزريًا. بالإضافة إلى ذلك ، قلل علاج سيليكوكسب بشكل كبير من التعبير عن جينات Bcl2 و VEGF في خلايا .HepG2 ولم يتم الكشف عن مستقبلات COX2 في جميع عينات خلايا .HepG2 كشفت نتائجنا أيضًا أن العلاج بمثبطات COX2 قلل بشكل كبير من عدد وحجم االمستعمرات التي تكونها خلايا .HepG2 . الخلاصة: قللت مثبطات COX2 من نمو خلايا HepG2 وكذلك جعلتها حساسة اكثر لدوكسوروبيسين ورالوكسيفين. وتم الكشف على أن قدرة سيليكوكسيب على التقليل من نمو الخلايا يكون من خلال تقليل التعبير الجيني لكل من جيني Bcl2 و VEGF. بينما لم يتم الكشف على وجود جين COX2 في خلايا HepG2 لذلك يبدو ان مثبطات COX2 تعمل على تقليل نمو خلايا HepG2 من خلال اليات اخرى غير تثبيط مستقبلات COX2 وايضا لوحظ انخفاض كبير في تكوين المستعمرات الناتجة عن خلايا HepG2 عند استخدام مثبطات COX2 بتركيز مكافئ لـ IC50.

الكلمات الدالة: سرطان الخلايا الكبدية، مثبطاتCOX2 ، سيليكوكسب.

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المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

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

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

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

والله ولي التوفيق

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