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Jordan Journal of PHARMACEUTICAL Sciences

Specialized International Referreed Journal

Issued by the Scientific Research Support Fund

This title is indexed in Scopus

مجلد (15) العدد (3)، أيلول 2022

Volume 15, No. 3, September 2022

Established 2007

ISSN: 1995-7157

EISSN: 2707-6253

Publisher

The University of Jordan
Deanship of Scientific Research
Amman 11942 Jordan
Fax: +962-6-5300815

National Deposit
(23.3/2008/D)
(Journal's National Deposit Number at the Jordanian National Library)

Jordan Journal of Pharmaceutical Sciences

Volume 15, Number (3), ***September*** **2022**

JORDAN JOURNAL OF PHARMACEUTICAL SCIENCES

A RESEARCH INTERNATIONAL SPECIALIZED REFEREED JOURNAL
ISSUED BY: SCIENTIFIC RESEARCH SUPPORT FUND AND THE UNIVERSITY OF JORDAN

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Scopes:

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

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Book

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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

We all hope that this year would be the end of the pandemic, so life will start again. We started -although slowly- getting back to normal life. Teaching and meetings are again face to face, and researchers are again working together. Jordan Journal of pharmaceutical Sciences (JJPS) is not an exception; our editorial team enjoyed face to face discussions, selecting reviewers and taking decisions related to research works after those hard times working completely online before. JJPS continues to publish the 4 issues of (JJPS) on regular times: one issue every quarter with 10 accepted articles per issue. Despite the enthusiasm, ambition and optimistic teamwork of the editorial team, challenges are still being faced; particularly waiting time from submission till sending a decision to the researcher. One of the main obstacles that causes the delay is the electronic system of submission, tracing and evaluation, as most researchers, reviewers and editorial members are suffering from the current user-unfriendly system. Meetings of the Jordanian journals' Editors-in-chief with the administrative and technical people in the Deanship of Scientific Research led to a promise for introducing a completely new electronic system that will make life much easier for researchers, reviewers, editorial board members and even the editorial working team. The latter just finished its trial version with good feedback so far. JJPS people are looking forward to having this new faster system implemented soon hoping that the second issue for 2022 will be fully and easily practiced by all .

JJPS teams started already to classify reviewers according to their time of response to the reviewing process, working with (A) class reviewers would decrease times for researchers who submitted their work to the JJPS waiting for the feedback. In general, we have distinguished colleagues from more than 30 universities in Jordan representing all scientific pharmaceutical domains and with a diversified experience: recent comers from well-known high ranking world universities as well as wise experienced current available scientists .

The University of Jordan recently agreed its new financial budget for 2022; the good news is that the scientific research budget allocated this year is double than the previous year. Which hopefully would reflect on the quality of the research performed and subsequently published for the academicians in the region.

Prof Ibrahim Alabbadi
Editor-in-Chief

CONTENTS

ORIGINAL ARTICLES

Jordan Journal of Pharmaceutical Sciences, Volume 15, No. 3, 2022

Investigation of the Chemical Stability of Lenalidomide in Methanol/Ethanol Solvents Using RP-HPLC-UV and LC-MS

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[1]School of Pharmacy, The University of Jordan, Jordan.
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ABSTRACT

Lenalidomide is a heterocyclic drug used for the treatment of Myelodysplastic syndrome. The current research focuses on the structural elucidation of new degradants that are formed unexpectedly upon storage of lenalidomide in methanol, followed by proposing their corresponding formation mechanism. The proposed structures of the degradants are relatively stable in which two tetrahedral intermediates are resulted from nucleophilic addition of methanol to the carbon of the carbonyl group of imide ring. Methanol molecules, as a solvent, may contribute in stabilizing the intermediate via hydrogen bond formation with it. These degradants were found abundant in lenalidomide/ methanol solution. Hence, the toxicological evaluation of them is crucial.

Keywords: Lenalidomide, Degradants, RP-HPLC, Mass spectrometry.

INTRODUCTION

Lenalidomide [3-(4-amino-1-oxo 1,3-dihydro-2*H*-isoindol-2-yl) piperidine-2,6-dione] (Fig. 1) is a heterocyclic drug which is used for the treatment of Myelodysplastic Syndrome (MDS) [1,2]. Lenalidomide is a potent thalidomide analog showed fewer adverse effects than the potent drug thalidomide. Lenalidomide displayed promising results in phase II trials towards myelofibrosis and myelofibrosis with myeloid metaplasia[3]. Consequently and based on the success of the clinical studies, lenalidomide was approved by the US Food and Drug Administration (US-FDA) and registered by Celgene Corporation (New Jersey, USA) under the commercial name Revlimid® [4].

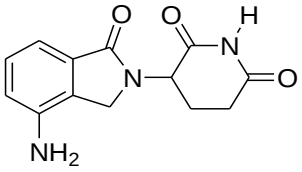

Fig. 1. Chemical structure of lenalidomide

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Received: 24/5/2021 Accepted: 28/12/2021.
DOI: *https://doi.org/10.35516/jjps.v15i3.406*

Identification of significant degradants and impurities in the active pharmaceutical ingredient (API) is mandatory by many regulatory authorities [5] and should be well-established since these impurities may affect the safety and efficacy of APIs [6,7]. Furthermore, some of the unidentified degradants could be hazardous and toxic even if they present in low quantities in the finished pharmaceutical formula [8,9]. Therefore, chemical structures of these substances should be well characterized. Few papers were reported for the determination of lenalidomide related impurities/degradants in the raw material or in the finished product. Lenalidomide-excipient blend from the capsule pharmaceutical dosage form was subjected to different ICH prescribed stress conditions of thermal stress, pH hydrolysis, oxidation and photolysis. The separations and characterization of components were achieved through a multi-step gradient elution using an HPLC with spectrophotometric and Mass techniques [10].

A new chromatographic method was established for the determination of lenalidomide and its related substances in capsules using Sunfire C-18 column with 85:15 v/v ratio of mobile phases A (mixture of phosphoric acid buffer and 1-octane sulphonic acid sodium salt) and B (55: 45 v/v ratio of methanol and acetonitrile) at 210 nm wavelength. The degradation studies were conducted using 0.1 M HCl, 0.1 M NaOH, 1% (v/v) H_2O_2 solutions, UV at 254 nm, Sun light, and heat to 60ºC. No significant degradation of lenalidomide was detected [11].

In continuation to our recent research in the respect of structural determination of new degradation products in various pharmaceutical ingredients [12–15], the current research is focusing on the investigation of the chemical stability of lenalidomide starting material in methanol and ethanol followed by structural elucidation of new degradants that are formed unexpectedly upon storage of lenalidomide in the former organic solvents, followed by proposing their corresponding formation mechanisms. Methanol and ethanol (protic organic solvents) are highly employed in the literature for preparing various lenalidomide solutions. Establishment a new analytical method for the assay determination of lenalidomide is not the scope of the present work.

Experimental

Chemicals and reagents

Lenalidomide raw material (purity 98.5%) was obtained from Reliance Chemical (Pomba, India). HPLC quality deionized water, acetonitrile, methanol, ethanol, formic acid were purchased from Merck (Darmstadt- Germany).

Instrumentation

HPLC system: Dionex ultimate 3000 for instrument equipped with a Diode array Detector DAD-RS 3000 (Dionex-Germany), LC pump (model), auto sampler (model), column oven, and windows 7-Chromeleon 7.2 software chromatography data system. LC-MS, LC: (Agilent 1200), mass detector: API 3200-AB Sciex triple quad, ESI ionization technique and ultra violet lamp (Chromato-Vue-C-70G). The applied analytical method was taken from the publication for Shu *et al* and modified to suit this work [16]. Mobile phase was composed of the formic acid solution (pH 3.0) and acetonitrile (90:10 v/v), respectively, then filtered through 0.45 µm nylon membrane filter and degassed. Inertsil ODS-3V column (25 cm length, 4.6 mm internal diameter, 5 µm particle size) at 25 °C was used as analytical column. The mobile phase was kept at flow rate 1.0 ml/min and the injection volume was 10 µl. Mettler toledo pH meter FP20 and Elma ultrasonic bath S30.

Preparation of Lenalidomide standard solution

10 mg of lenalidomide raw martial was dissolved in 10 ml of acetonitrile to give a concentration of 1.0 mg/ml.

Time-dependent degradation in methanol (or ethanol)

20 mg lenalidomide raw material was mixed with 20 ml methanol (or ethanol) to give a concentration of 1.0 mg/ml, this solution was kept at room temperature in dark place for 10 days. Then, the sample solution was filtrated using 0.45µm Nylon syringe filter.

Heat-dependent degradation in methanol

20 mg lenalidomide raw material was mixed with 2 ml methanol, this solution was then refluxed for 2 days at 70 °C in water bath and diluted with methanol to give a concentration of 1.0 mg/ml. Then, the sample solution was filtrated using 0.45µm Nylon syringe filter.

Results and Discussion

Recently, many published papers examined the chemical stability of lenalidomide starting material towards different stress conditions and by using various HPLC methods but none of them identified the structures of any developed degradants [17–19].

In the present work, a methanolic solution of lenalidomide starting material was prepared at room temperature followed by successive analysis for 10 days using a validated HPLC method [16]. HPLC chromatogram (Fig. 2, A) shows two significant degradants at RRTs 1.3 and 1.8 with a relative intensity of (1:3) on the tenth day. None of these new degradants belongs to any of the known impurities [20].

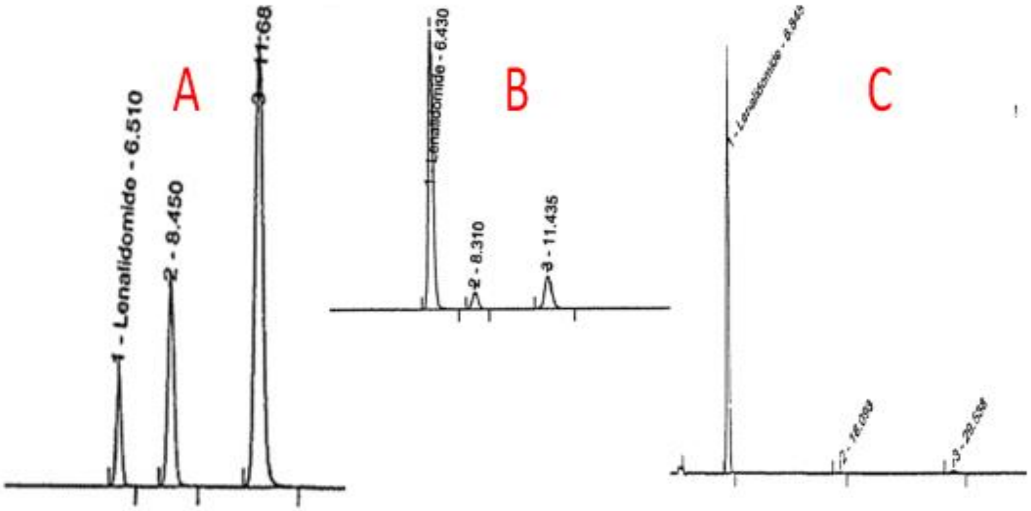


Fig. 2. HPLC Chromatogram of methanolic solution of lenalidomide stored at room temperature for 10 days (A), methanolic solution of lenalidomide refluxed for 2 days (B) and ethanolic solution of lenalidomide stored at room temperature for 10 days (C). The first peak in each chromatogram for lenalidomide.

To investigate the effect of heat on the formation of these corresponding degradants, a methanolic solution of lenalidomide was refluxed for 2 days then analyzed by employment the same analytical method, the corresponding HPLC chromatogram (Fig. 2, B) shows the same two degradants (same RRT's) but depicted lower area percent 6 and 15%, respectively.

Other organic solvents (ethanol, higher alcohols and acetonitrile) were employed to examine the effect of these solvents on the chemical stability of lenalidomide but unfortunately, lenalidomide has a limited solubility in higher alcoholic solvents. However, a suspended solution of lenalidomide in ethanol was prepared and stored at room temperature for 10 days. HPLC chromatogram (Fig. 2, C) shows two degradants at RRT's 2.64 and 4.31 resulted with area percent 0.37 and 1.71%, respectively. It is interesting to

note that the area percent of lenalidomide was 97.91% which may indicate that the response factors of both degradants are almost identical. On the other hand, a solution of lenalidomide in acetonitrile, which was prepared and stored for the same time interval showed no degradants at the specified retention times (after the peak of lenalidomide).

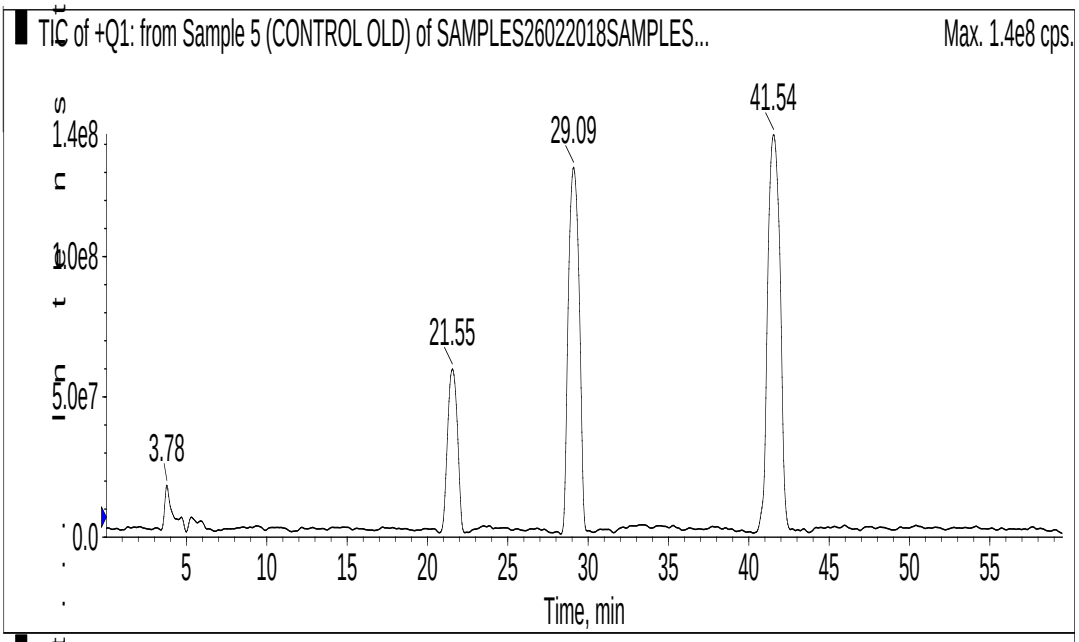
LC-MS analysis was employed to determine the masses of these interesting degradants. The proton adduct of the molecular ion of lenalidomide is 260 m/z, based on the positive mode of analysis. Other relevant lenalidomide peaks have the masses of 282 and 298 which belong to $[M+Na]^+$ and $[M+K]^+$, respectively. It is interesting to note that the molecular masses of both degradants is the same (*m/z* 292), in addition, two extra peaks are also observed at *m/z* 314 and 330 due to the formation of sodium and potassium adducts (Fig. 3). The fragmentation pattern of MS spectra is similar for these two degradants.

From the polarity point of view, lenalidomide and its two degradants are significantly different in this respect. Under the present analytical conditions, lenalidomide has the shortest retention time and therefore considered the most polar one (predicted *log p* -0.83). While, other degradants (RRTs 1.3 and 1.8) have predicted *log P* values -0.14 and 0.02, respectively. Based on the MS and polarity data, chemical structures of these degradants were proposed as shown in Fig. 4 and their names as 4-amino-2-(2-hydroxy-2-methoxy-6-oxopiperidin-3-yl)isoindolin-1-one (**A**) and 4-amino-2-(6-hydroxy-6-methoxy-2-oxopiperidin-3-yl)isoindolin-1-one (**B**).

We supposed that these degradants are formed by the mechanism of nucleophilic addition of methanol to the carbonyl group; other related chemical structures were examined but excluded since none of them is completely compatible with obtained experimental data in terms of molar masses and polarity. For example, ring opening products of the intermediates in Fig. 4 were proposed since their molar masses are *m/z* 292 but they were excluded since their predicted *log P* values were -0.82 for both structures (closer to the *log P* value of lenalidomide) which means that they should have retention times closer to that of lenalidomide. In addition, ring opening reactions cannot be produced unless lenalidomide is hydrolyzed under vigorous experimental conditions since cyclic imide group is relatively stable.

Interestingly, in rhodium catalyzed carbomination of substituted alkene, it has been found that the usage of methanol as a solvent is crucial for improving the efficiency of the reaction relying on the reactivity of enoxyphthalimide [21]. The latter is an imide ring resembles piperidone-2,6-dione part of lenalidomide (Fig.1). Deep insight for the methanol reactivity towards the imide ring was introduced by Chen *et al.* [22], they found that methanol-assisted ring opening of imide group in phthalimide undergoes stepwise mechanism rather than a concerted opening. Their calculations justified the occurrence of a relatively stable tetrahedral intermediate resulted from the attack of imide ring by a methanol molecule before the ring is opened. A structure similar to our proposed degradants in Fig.4 which represents the tetrahedral intermediates of methanol addition to piperidone-2,6-dione part of lenalidomide. According to Chen *et al* calculations, the energy barrier of the concerted ring opening of phthalidimide is quite high whereas the barriers of stepwise mechanism is lesser and more acceptable in justifying the reaction occurrence at room temperature. Moreover, it has been reported that the activation energy of tetrahedral intermediate is almost similar to the activation energy of phthalimide opening from the intermediate [22]. Therefore, these calculations justified the relative stability and occurrence of our suggested degradants of lenalidomide (Fig. 4). Additionally, using methanol as a solvent might participate in stabilizing the tetrahedral intermediates due to excessive hydrogen bonding formation between methanol as a solvent and the methoxy and hydroxyl groups of the degradants.

A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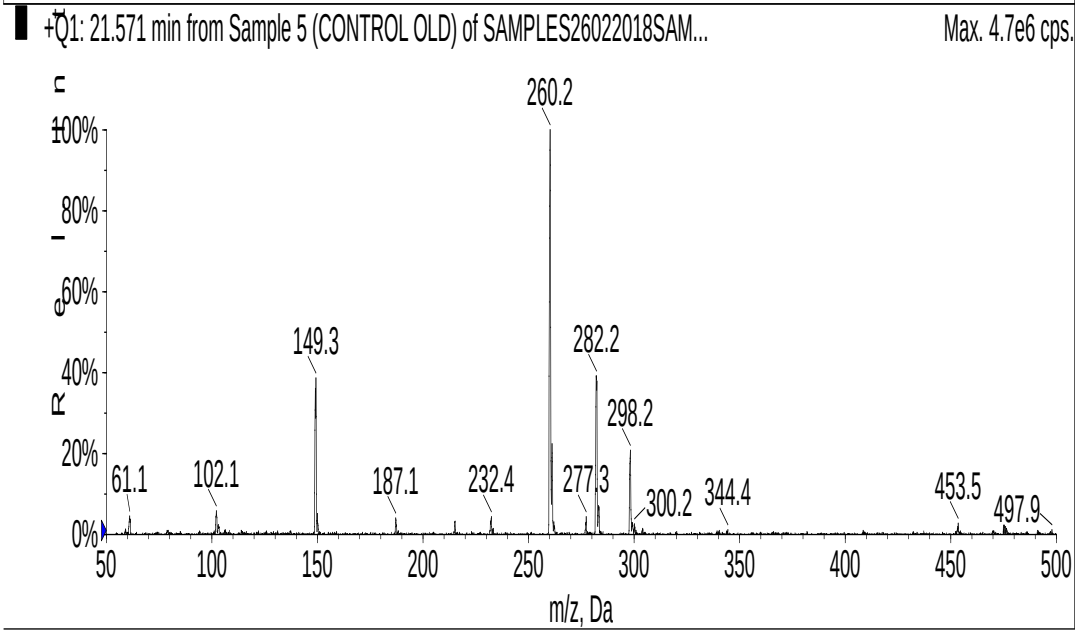
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277.3
282.2
298.2
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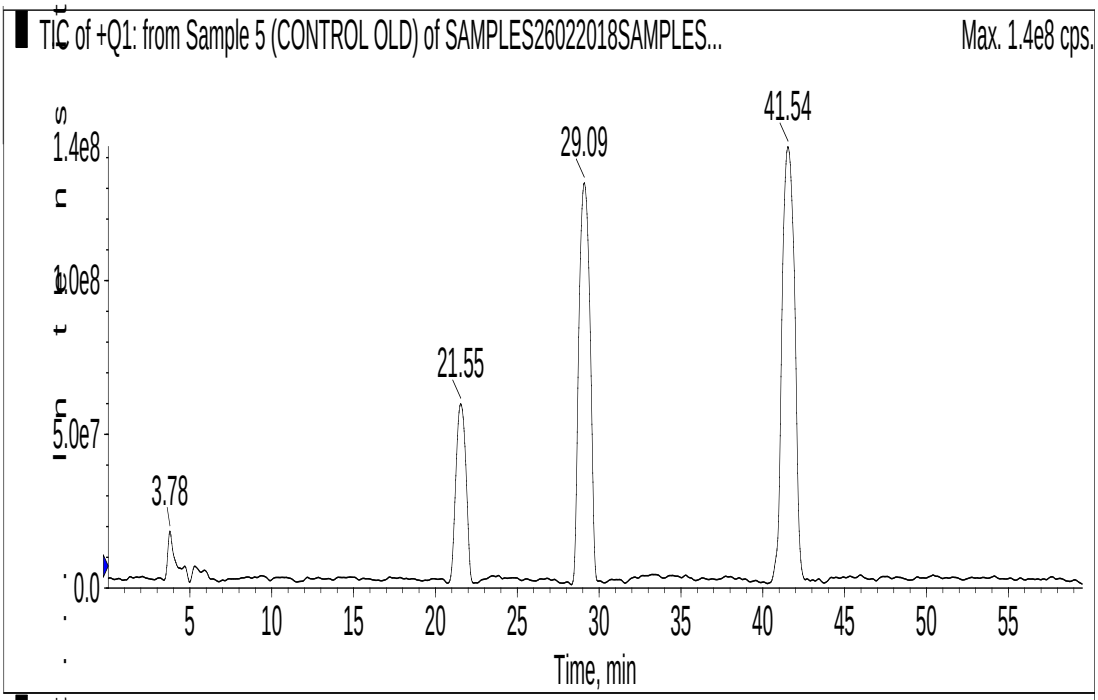
m/z, Da

B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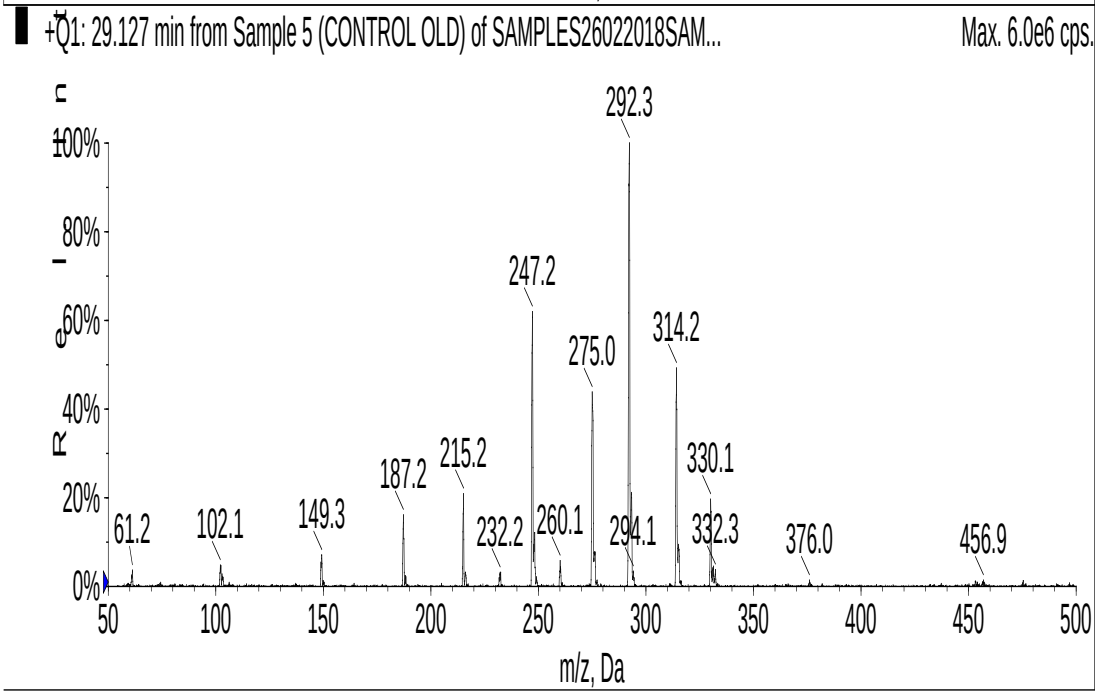
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260.1
275.0
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314.2
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C

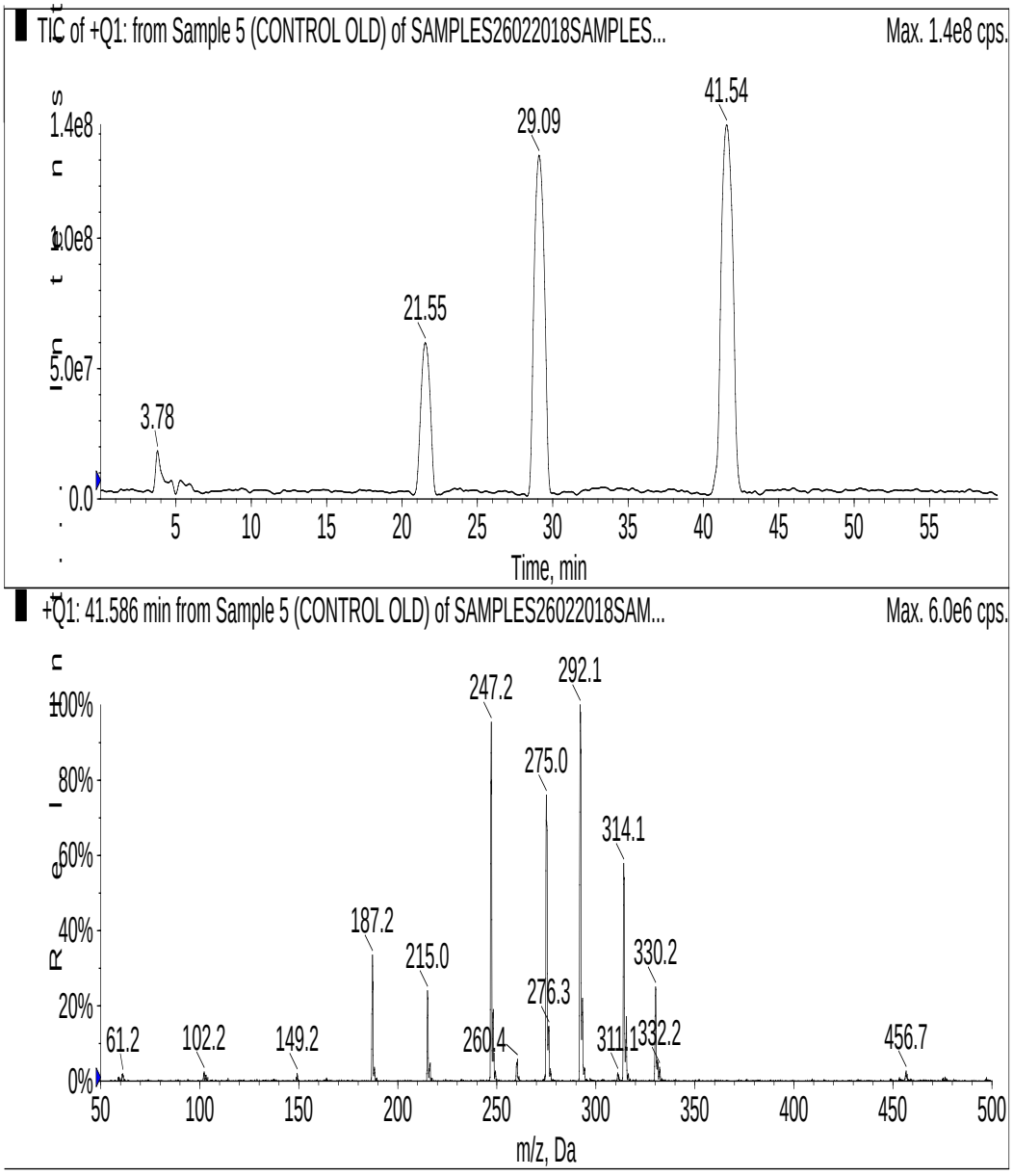

Fig. 3. LC-MS chromatograms and MS charts for degradants at RTs 29.1and 41.5 min. (A) denotes Lenalidomide chromatogram and mass chart.

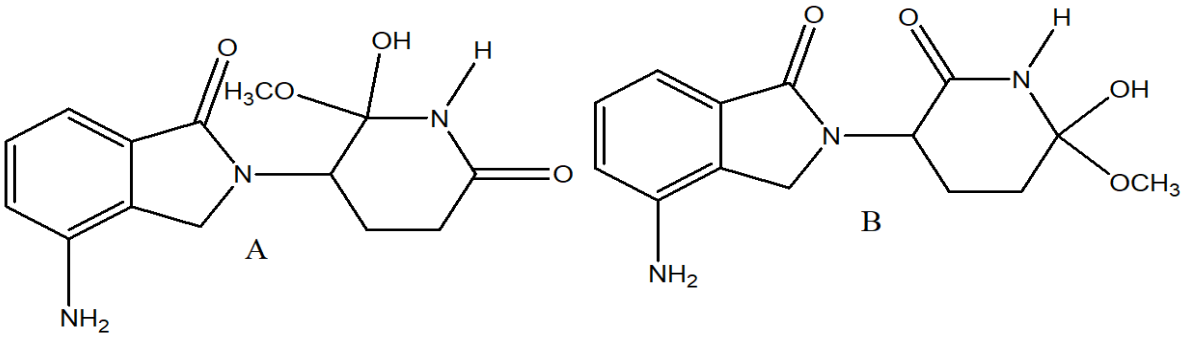

Fig. 4. Proposed chemical structures of the two degradants A and B (RRTs 1.3 and 1.8, respectively).

Conclusions

Two new degradants have been detected when methanolic solution of lenalidomide is prepared and kept at room temperature accomplished with increasing their amounts with time. It is highly advisable to monitor these degradants in various pharmaceutical samples (e.g. stability samples) in case alcoholic solvents, particularly methanol, are employed during the formulation and analysis processes.

Acknowledgements

Authors gratefully acknowledge the financial support from the University of Jordan, Deanship of Scientific Research.

List of abbreviations

LC-MS	**Liquid chromatography-mass spectrometry**
API	Active pharmaceutical ingredient
API	Atmospheric pressure ionizer
ESI	Electrospray ionizer
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MDS	Myelodysplastic Syndrome
US-FDA	United states-food and drug administration
ODS	Octadecyl silane
$[M+Na]^+$	Sodium adduct of molecular ion
$[M+K]^+$	Potassium adduct of molecular ion
M / Z	Mass over charge
MS	Mass spectrometry
RRT	Relative retention time

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التحقق من الاستقرار الكيميائي لليناليدوميد في مذيبات الميثانول / الإيثانول باستخدام الطور العكسي للكروماتوغرافيه والكروماتغرافيه السائلة مع مطياف الكتلة

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ملخص

لينااليدوميد دواء غير متجانس الحلقة يستخدم لعلاج متلازمة ميلادوبلاستيك. يركز البحث الحالي على الشكل البنيوي للمواد المتحللة الجديدة التي تتشكل بصوره غير متوقعه عند تخزين اللينااليدوميد في الميثانول، متبوعًا باقتراح آلية تكوينها. الاشكال المقترحة للمواد المتحللة مستقرة نسبيًا حيث ينتج وسيطان رباعي السطوح من إضافة ميثانول محبة للنواة إلى كربون مجموعة الكربونيل في حلقة الإيميد. قد تساهم جزيئات الميثانول، كمذيب، في تثبيت المادة الوسيطة عبر تكوين رابطة الهيدروجين معها. تم العثور على هذه المواد المتحللة بشكل وفير في محلول لينااليدوميد/ ميثانول. لذا فإن التقييم السمي لها أمر يعتبر بالغ الأهمية.

الكلمات الدالة: لينااليدوميد، المواد المتحللة، الطور العكسي للكروماتوغرافيه، مقياس الطيف الكتلي.

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تاريخ استلام البحث 2021/5/24 وتاريخ قبوله للنشر 2021/12/28.

Quantitative Structure Activity Relationship (QSAR) Investigations and Molecular Docking Analysis of Plasmodium Protein Farnesyltransferase Inhibitors as Potent Antimalarial Agents

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ABSTRACT

The development of *farnesyltransferase* inhibitors based on the benzophenone scaffold directed against *Plasmodium falciparum* is considered a strategy in malaria treatment. In this work, quantitative structure–activity relationship (**QSAR**) was performed to predict the protein *farnesyltransferase* (**PFT**) inhibitory activities for a series of 36 benzophenone derivatives. The data set was divided into two subsets of training and test sets, and the best model using **multiple linear regression** (**MLR**), with the values of internal and external validity (R^2 = 0.884, R^2adj = 0.865, R^2pred = 0.821, Q^2cv =0.822 and R^2p=0.811) was found in agreement with the Tropsha and Golbraikh criteria. The applicability domain (AD) was determined using the Williams plot to describe the chemical space for the model used in this study. The model shows that antimalarial activities of benzophenone depend on logP, bpol, MAXDn, and FMF descriptors. These indications prompted us to design new benzophenones PFT inhibitors and predict the value of their anti-malarial activities based on the MLR equation. Docking results reveal that the newly designed benzophenones bind to the hydrophobic pocket and polar contact with high affinity. The predicted results from this study can help to design novel benzophenone as inhibitors of human **PFT** with high antimalarial activities.

Keywords: QSAR, docking, benzophenone, PFT inhibitory, antimalarial.

I. INTRODUCTION

Malaria is one of the most important infectious diseases in the world [1]; it affects 400–900 million people each year in the world and is also the cause of death of about one to three million people annually [2]. Malaria can be caused by several species of **Plasmodium (**P**)** parasites, each of which has a complex life cycle: Plasmodium vivax, P. ovale, P. malaria, and P. falciparum. [3].

Plasmodium falciparum causes a more severe type of malaria, with a greater risk of mortality for individuals who get it [3]. It causes severe and/or lethal complications including cerebral malaria defined as coma, altered mental status, or multiple seizures. Recent efforts at the control of *falciparum* malaria have generally been unsuccessful, in large part due to the continuous development of *P. falciparum*'s resistance to conventional antimalarial [4]. Thus, there is a great need for the identification of new agents against multi-drug resistant *Plasmodium* and the evaluation of potential new compounds that act as an inhibitor for the treatment of malaria. Inhibitors of the protein *farnesyltransferase* (PFT) enzyme have emerged as a promising target for treating malaria caused by the Plasmodium *falciparum* parasite. *Farnesyltransferase* is a

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Received: 17/9/2021 Accepted:12/1/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.407*

catalytic enzyme that catalyzes the transfer of a farnesyl residue from farnesyl diphosphate to the thiol of a cysteine side chain of proteins carrying the CAAX-tetra peptide sequence (C: cysteine, A: aliphatic amino acid, X: serine or methionine) at their C terminus [5, 6]. PFT inhibitors are promising drugs for the treatment of malaria, and a number of different scaffolds have been shown to inhibit the growth of the malaria parasite *in vitro* and *in vivo* [7]. Previously, a class of *farnesyltransferase* inhibitors based on a benzophenone scaffold has developed by Wiesner and his co-workers, to find new synthetic inhibitors with simple structure and low-cost properties [8]. It was observed that compounds of this type suppress the growth of the multiresistant **Plasmodium** *falciparum* strain Dd2 in the nanomolar range [8].

In order to find new leads in the process of drug design and discovery, it would be helpful to examine chemical databases and virtual libraries against molecules with known activities or properties by using computational procedures. For this purpose, analysis of the relationship between structure and quantitative activity is widely used [9]. The Two-Dimensional Quantitative Structure-Activity Relationship (2D-QSAR) is a useful tool for describing the relationships between chemical structure and experimental data [10]. The relationship can be used to achieve the modeler's intended purpose, such as increasing the efficiency of an operation, lowering the toxicity of dangerous substances, or improving the pharmacological activity of pharmaceuticals, every phase of the predictive QSAR modeling analysis involves the application of multiple mathematical principles, and a huge quantity of quantitative data is created. The main purpose is to convert chemical information into useful numbers (descriptors), then build a mathematical link with the response [11]. The descriptors for 2D QSAR can be categorized according to their nature as well as calculation method, such as constitutional, topological, geometrical, electrostatic, quantum-chemical, and thermodynamic descriptors [12].

The present work describes the descriptor-based QSAR studies developed for a series of benzophenone derivatives that were previously reported in the literature and evaluated for their antimalarial activity. In addition, to predict the activity of newly designed compounds based on their molecular properties, we used the 2D-QSAR model by altering molecular descriptors and chemical fragments, which were determined to be important within the model's applicability scope. Furthermore, we explored the binding interactions of the newly designed benzophenone derivatives with protein *farnesyltransferase* receptor, to provide new information that might be useful in the development of new inhibitors with improved antimalarial properties.

II. DATA SET AND COMPUTATIONAL METHODS

1. *Data set for analyze*

The **2D-QSAR** studies performed on 36 benzophenone derivatives were obtained with their anti-antimalarial activities against **Dd2** strain of **Plasmodium** *falciparum* from the work of Wiesner and co-workers [8, 13-16]. After conversion of the IC50 values to micromole, we generated the pIC_{50} values for each of the 36 compounds using the following: $pIC50 = -\log(IC50)$ (1)

The **2D** structures of the molecules were prepared using Marvin Sketch (https://www.chemaxon.com) [17], and converted to **3D** and optimized the molecular geometry using HyperChem software [18], as shown in Table 1. The geometries of benzophenone derivatives were first fully optimized by molecular mechanics, with MM^+ force-field (RMS = 0.001 Kcal/Å). Then, geometries were fully re-optimized by using PM3 method.

2. Calculation of molecular descriptors

The PaDEL descriptor [19] was used to calculate a pool of descriptors of the optimized molecules of benzophenone derivatives. The constitutional, autocorrelation, Basak, BCUT, Burden, connectivity, E-state, Kappa, extended topochemical atom (ETA), molecular property, and topological descriptors were computed. Among these descriptors, 19 descriptors were

selected and the other descriptors were eliminated after pretreatment on the basis of the correlation coefficient cut-off value of 0.80 (Tables S1 and S2) and the variance cut-off value of less than 0.0001, as presented in Table 2.

3. Data Splits and Model Development

The data set was divided into two subsets, the training data set and the test data set, the division was random by the software. Training set (70%) was used in building the model while test set (30%) in validating the model.

In this stage, the multiple linear regression (**MLR**) analysis of the training set was carried out using XLSTAT software between a response variable Y (pIC50) and independent variables X (2D) molecular descriptors to find a linear model of the activity of interest, which takes the form of the multiple linear regression equation [20].

The best model **QSAR** was chosen based on the statistical validation parameters like the correlation coefficient of determination (**R^2**), Adjusted correlation coefficient **R^2** (**R^2adj**), predicted residual sum of squares (***PRESS***), total sum of squares (***SSY***) and standard deviation based on predicted residual sum of squares (***S*PRESS**) [21] all are represented in Eqs. (2), (3), (4) and (5):

$$R^2_{adj} = 1 - (1 - R^2)\frac{n-1}{n-p-1} = \frac{(n-1)R^2 - p}{n-p+1} \quad (2)$$

$$PRESS = \sum_{i}^{i=n} (y_i - \hat{y}_i) \quad (3)$$

$$SSY = \sum_{i}^{i=n} (y_i - \bar{y}) \quad (4)$$

$$S_{PRESS} = \sqrt{\frac{\sum_i^{i=n} (y_i - \hat{y}_i)^2}{n-p-1}} = \sqrt{\frac{PRESS}{n-p-1}} \quad (5)$$

Where:

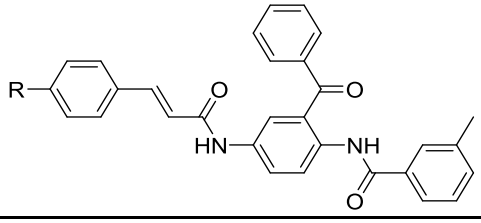
y_i is the observed activity of the training set compounds

$\hat{y}_i$ is the predicted activity of the training set compounds.

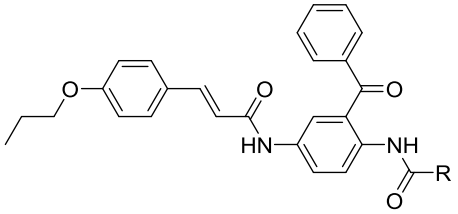
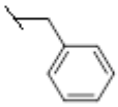
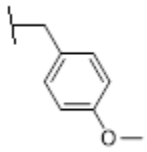
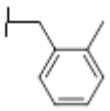
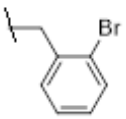
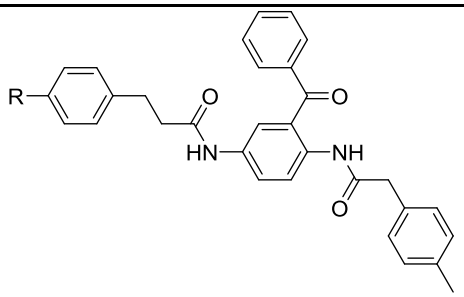
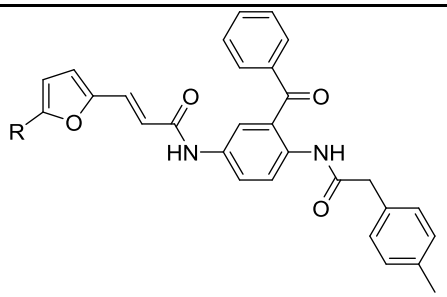
$\bar{y}$ is mean observed activity of the training set compounds and n number of objects.

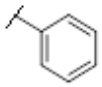
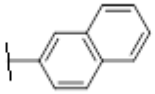
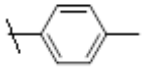
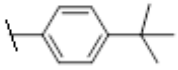
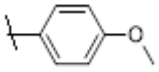
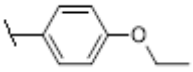
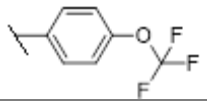
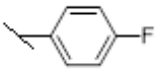
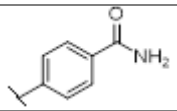
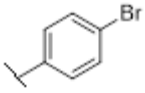
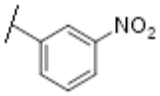
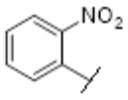
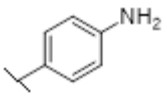
p number of predictor variables.

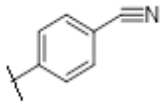
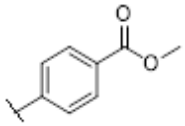
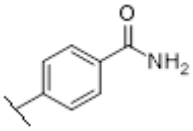
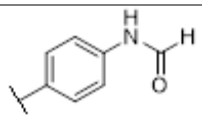
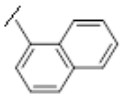
Table 1: Chemical structures and PFT inhibitors activities of benzophenone derivatives



Comp. No	R	pIC50exp µM	pIC_{50}pred µM	Residue
1	-H	5.24	5.234	0.006
2	-Cl	5.26	5.396	-0.136
3t	$-NO_2$	5.19	5.318	-0.128
4	-Br	5.49	5.598	-0.108
5	$-NH_2$	5.26	5.314	-0.054
6t	$-CH_3$	5.85	5.568	0.282
7	$-CF_3$	5.24	5.560	-0.320
8	$-O-CH_3$	5.89	5.730	0.160
9	$-CH_2-CH_3$	5.92	5.641	0.279
10	$-CH(CH_3)_2$	5.92	5.590	0.330
11	$-C(CH_3)_3$	5.52	5.641	-0.121

12	-O-CH_2-CH_3	6.07	5.780	0.290
13	-O-$(CH_2)_2$-$(CH_3)_3$	5.96	5.847	0.113
				
14		5.60	5.552	0.048
15		5.89	5.848	0.042
16t		5.62	5.719	-0.099
17		5.55	5.671	-0.121
				
18	H	5.57	5.658	-0.088
				

19t		6.38	6.148	0.232
20t		6.70	6.664	0.036
21t		6.92	6.534	0.386
22t		6.52	6.833	-0.313
23		6.89	6.754	0.136
24		6.52	6.664	-0.144
25		6.68	6.736	-0.056
26		6.49	6.505	-0.015
27		6.25	6.589	-0.339
28		6.90	6.492	0.408
29		6.23	6.248	-0.018
30		6.17	6.248	-0.078
31		6.59	6.568	0.022

32		6.66	6.619	0.041
33		6.77	6.642	0.128
34t		6.25	6.289	-0.039
35		6.55	6.502	0.048
36		6.00	5.672	0.328

t: Test set compounds.

Table 2: Values of parameters calculated for the studied compounds

Comp.	ALogP	ALogp2	AMR	apol	bpol	Lipa	MAX DP	DEL S2	FMF	MAX DN	VAB C	VAdj Mat	MW	AM W	WPA TH	WPOL	XLogP	Zagreb	TPSA
1	-0.49	0.245	40.5	76.5	32.6	12.3	6.315	36.41	0.51	1.992	457.8	6.169	474	7.64	4550	52	11.43	182	75.3
2	0.35	0.123	46.2	76.4	29.0	11.7	6.350	43.49	0.54	1.977	471.0	6.209	528	8.95	4956	54	9.19	188	75.3
3	0.16	0.027	44.4	78.5	32.7	10.6	6.342	51.61	0.50	2.496	483.8	6.285	519	8.11	5844	58	10.26	198	118.4
4	0.39	0.154	49.1	78.8	32.8	12.2	6.349	37.88	0.51	1.986	477.1	6.209	552	8.90	4956	54	10.81	188	75.3
5	-1.24	1.551	44.7	78.2	33.0	11.4	6.328	39.92	0.50	2.001	468.8	6.200	489	7.64	4956	54	9.35	188	101.2
6	0.15	0.021	46.0	79.5	34.8	12.7	6.342	36.68	0.49	1.990	475.1	6.209	488	7.51	4956	54	11.75	188	75.3
7	0.60	0.363	46.9	79.2	35.1	13.9	6.333	64.67	0.49	5.693	493.3	6.321	542	8.34	6291	60	11.91	206	75.3
8	-0.54	0.299	47.8	80.3	36.7	12.2	6.357	40.04	0.48	2.018	483.9	6.247	504	7.63	5399	56	9.93	192	84.5
9	-0.18	0.035	50.8	82.6	36.9	13.2	6.367	37.22	0.47	1.987	492.4	6.247	502	7.38	5399	56	12.32	192	75.3
10	0.08	0.005	54.7	85.7	39.1	13.9	6.390	37.86	0.45	1.988	509.7	6.285	516	7.27	5844	58	12.80	198	75.3
11	1.06	1.125	59.6	88.8	1.06	14.7	6.413	38.49	0.43	1.991	527.0	6.321	530	7.10	6291	60	13.43	206	75.3
12	-0.29	0.088	52.1	83.4	38.9	12.6	6.378	40.82	0.46	2.015	501.2	6.285	518	7.51	5880	57	10.35	196	84.5
13	-1.16	1.351	58.5	89.6	43.2	13.8	6.415	41.85	0.42	2.011	535.8	6.357	546	7.28	6960	59	11.28	204	84.5
14	-1.24	1.549	55.9	86.5	41.0	13.3	6.462	42.30	0.43	2.015	518.5	6.321	532	7.39	6210	61	10.86	200	84.5
15	-1.29	1.680	63.2	90.4	45.1	13.3	6.542	46.59	0.40	2.028	544.6	6.392	562	7.39	7039	65	9.79	210	93.7
16	-0.60	0.363	61.4	89.6	43.2	13.8	6.516	42.87	0.41	2.015	535.8	6.357	546	7.28	6600	64	11.19	206	84.5
17	-0.35	0.126	64.5	88.9	41.2	13.2	6.525	44.26	0.43	2.008	537.8	6.357	610	8.47	6600	64	10.45	206	84.5
18	-0.75	0.576	39.6	77.8	34.8	11.8	6.329	36.83	0.50	1.901	460.5	6.169	476	7.44	4550	52	11.17	182	75.2
19	-0.06	5.775	47.6	88.8	38.9	14.0	6.440	42.24	0.51	2.057	524.6	6.392	554	7.69	7278	62	12.08	218	88.4
20	-0.07	5.775	47.6	97.1	41.1	16.1	6.518	44.10	0.52	2.061	565.2	6.523	604	7.74	9358	72	13.95	244	88.4
21	0.63	0.402	53.1	91.8	41.0	14.5	6.466	43.03	0.49	2.056	541.9	6.426	568	7.57	7705	65	12.40	224	88.4
22	0.19	0.036	59.2	95.7	45.1	14.5	6.495	47.06	0.46	2.072	568.0	6.491	598	7.57	8829	68	11.42	232	97.6
23	-0.05	0.003	54.9	92.6	43.0	14.0	6.478	46.27	0.48	2.074	550.7	6.459	584	7.68	8245	67	11.00	228	97.6
24	1.55	2.398	66.7	101.0	47.6	16.6	6.521	45.17	0.44	2.057	593.8	6.523	610	7.26	9331	71	14.07	242	88.4
25	0.08	0.006	54.6	92.5	43.1	14.1	6.477	54.53	0.48	2.433	556.7	6.491	602	7.92	8829	68	11.27	232	97.6
26	0.47	0.222	48.5	88.6	39.0	14.2	6.443	53.34	0.51	2.116	530.6	6.426	572	7.94	7705	65	11.40	224	88.4
27	-0.63	0.400	60.5	96.1	43.8	13.7	6.502	52.68	0.46	2.109	576.3	6.523	611	7.73	9310	69	10.90	238	117.0
28	0.88	0.776	56.2	91.1	39.1	14.0	6.471	44.14	0.51	2.044	543.9	6.426	632	8.77	7705	65	12.09	224	88.4
29	-0.02	5.904	64.1	93.4	43.4	11.6	6.477	60.91	0.47	2.402	566.7	6.491	603	7.73	8749	69	10.87	234	12.0
30	-0.02	5.904	64.1	93.4	43.4	11.8	6.474	60.07	0.47	2.402	566.7	6.491	603	7.73	8851	69	10.45	234	127.0
31	-1.43	2.057	64.4	93.2	43.7	12.8	6.471	47.54	0.47	2.017	551.7	6.426	573	7.34	7705	65	9.96	224	110
32	-0.64	0.422	66.4	93.6	42.8	12.8	6.481	50.08	0.48	2.042	563.8	6.459	583	7.57	8210	67	10.54	228	108
33	0.00	1.296	59.6	95.2	43.9	13.4	6.499	55.89	0.47	2.148	574.1	6.523	612	7.84	9269	71	11.42	238	114
34	-0.64	0.412	55.0	93.1	40.2	12.8	6.480	55.15	0.48	2.236	559.0	6.491	597	7.85	8717	69	10.37	234	131
35	-0.71	0.505	56.4	93.1	41.6	13.2	6.484	50.88	0.48	2.110	559.0	6.491	597	7.85	8759	68	10.66	232	117
36	-0.48	0.234	52.5	82.9	37.6	11.7	6.379	48.37	0.47	2.107	507.4	6.321	532	7.82	6328	60	10.35	202	101

4. Model Validation

A QSAR study's major aim is to create a model with the best predictive and generalization abilities [22]. This is done to test the internal stability and predictive ability of the **QSAR** models, in this paper two principal type of validation (internal validation and external validation) were performed [23].

Common method for internally validating **QSAR** models is cross-validation (**CV**, Q^2, q^2, or jack-knifing). The CV method repeats the regression on subsets of data multiple times. Usually each molecule is left out once (only), in turn, and the Q^2 is computed using the predicted values of the missing molecule. CV is frequently used to calculate the maximum size of a model that may be utilized for a particular data collection. A cross-validated Q^2 is usually smaller than the overall R^2 for a **QSAR** equation. It's a diagnostic tool for determining an equation's predictive capability. The cross-validation regression coefficient (**Q^2cv**) was calculated with the following equation [18]:

$$Q_{cv}^2 = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y}_i)} = 1 - \frac{PRESS}{SSY} (6)$$

It has been reported that high estimation of statistical attributes is not enough to justify the ability of a model, and so to assess the predictive capacity of the new **QSAR** model, the method was used by Golbraikh and his co-workers [24], Roy and his co-workers [25].

The coefficient of determination for the test set R^2 test and other statistical characteristics of the test set are represented in Eqs. (7-11).

$$R_{pred}^2 = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y}_i)^2} (7)$$

$$R^{\circ 2} = 1 - \frac{\sum_{i=1}^{ntest}(\hat{y}_i - y_i^{r^\circ})^{k2}}{\sum_{i=1}^{ntest}(\hat{y}_i - \bar{\hat{y}})^2} . y_i^{r^\circ} = K\hat{y}_i (8)$$

$$R'^{\circ 2} = 1 - \frac{\sum_{i=1}^{ntest}(y_i - \hat{y}_i^{r^\circ})^2}{\sum_{i=1}^{ntest}(y_i - \bar{y})^2} . \hat{y}_i^{r^\circ} = K' y_i (9)$$

$$K = \frac{\sum_{i=1}^{ntest} y_i \hat{y}_i}{\sum_{i=1}^{ntest} \widehat{y^2}_i} (10)$$

$$K' = \frac{\sum_{i=1}^{ntest} y_i \hat{y}_i}{\sum_{i=1}^{ntest} {y_i}^2} (11)$$

Where **K** and **K'** are the slopes of regression lines through the origin for fits to experimental and predicted data respectively.

In addition, Roy and his co-workers [26] proposed a new simple external validation metric, as shown in the following equations:

$$r_m^2 = R^2(1 - \sqrt{|R^2 - R^{\circ 2}|}) \quad (12)$$

$$r_m'^2 = R^2(1 - \sqrt{|R^2 - R'^{\circ 2}|}) \quad (13)$$

$$\overline{r_{m(test)}^2} = \frac{r_m^2 + r_m'^2}{2} \quad (14)$$

This formula can be applied for both external and internal validation and the present study focuses on the external validation form.

In addition, we choose the concordance correlation coefficient (**CCC**) proposed by Lin [27] to measure the agreement between experimental and predicted data, which should be the real aim of any predictive **QSAR** models:

$$ccc = \frac{2\sum_{i=1}^{ntest}(y_i - \bar{y})(\hat{y}_i - \bar{\hat{y}})}{\sum_i^{ntest}(y_i - \bar{y})^2 + \sum_i^{ntest}(\hat{y}_i - \bar{\hat{y}})^2 + n_{test}(\bar{y} - \bar{\hat{y}})^2} (15)$$

5. Y-Randomization test

To establish model robustness, Y-randomization, randomization of the response variable, test was used this test consists of redoing all of the computations from the training set with scrambled activities. Calculations were repeated at least five times, to ensure reproducibility in the results, and after each iteration, a new **QSAR** model is developed [28].

New **QSAR** models had lower Q^2 and R^2 than those of the original models. This technique was performed to eliminate the possibility of random correlation. If higher values of Q^2 and R^2 are obtained, it means that an

acceptable **QSAR** cannot be generated for this dataset due to structural redundancy and random correlation.

Coefficient of determination, $\mathbf{cR^2p}$ value has been reported to be greater than **0.5** for passing this test, and it is also calculated in the Y-randomization test and is expressed as:

$$cR_P^2 = R_x(R^2 - R_P^2)^2 \quad (16)$$

Where **R** is the correlation coefficient for Y-randomization and $\mathbf{R^2r}$ is the average **'R'** of the random models [22].

6. Assessment of the applicability domain of the model

The reliability of a **QSAR** classification model depends on its capacity to achieve confident predictions of new compounds not considered in the building of the model [29]. The ability of a QSAR classification model to make credible predictions of novel compounds not addressed in the model's construction determines its dependability. The domain of applicability (**AD**) is an important concept in **QSAR** which makes it possible to estimate the uncertainty in the prediction of a new compound according to its similarity with the compounds used to build the model [30].

The Williams plot, the plot of standardized residuals versus leverage values (***h***), was used in the present study to visualize the **AD** of the **QSAR** model.

Leverage value of a given chemical compound ***i*** is defined as:

$$h_i = x_i^T(X^TX)^{-1}x_i \quad (17)$$

Where ***xi*** is the descriptor row-vector of the query compounds ***i***.

The warning leverage (***h****) is the limit of normal values for **X** outliers and ***i***. ***h**** is generally fixed at $\mathbf{3(k+1)/n}$ (**k** is the number of model parameters and **n** is the number of training set compounds), whereas **x = 2** or **3**. Prediction was considered unreliable for compounds with a high leverage value ($\mathbf{h > h^*}$). When a compound's leverage value is less than the threshold value, on the other hand, the agreement probability between observed and predicted values is as high as it is for the training set compounds [31-33].

7. Preparation of *farnesyltransferase* Protein and docking studies

Molecular docking calculations were carried out using MOE-Dock software [34]. The crystal structure of the protein farnesyltransferase (**PFT**) was obtained from the Protein Data Bank (PBD ID: 3E37) [35]. Crystal structure was edited to remove water molecules and was imported to into MOE, and then all hydrogen atoms were added to the structure followed by their optimization using Amber10: EHT force field. Active site was identified, as presented in Figure 1. The 3D structures of the ligands were optimized using MOE software with MMFF94x force field and Root Mean Square (**RMS**) gradient value of 0.001 kcal/mol Å. The ligand database that was developed form the total set of 11 newly designed compounds were used for docking with the known **PFT** receptor active site. Thirty ligand−receptor complex conformations were generated for each test compound, and the conformation with the least docking score was considered for further analysis.

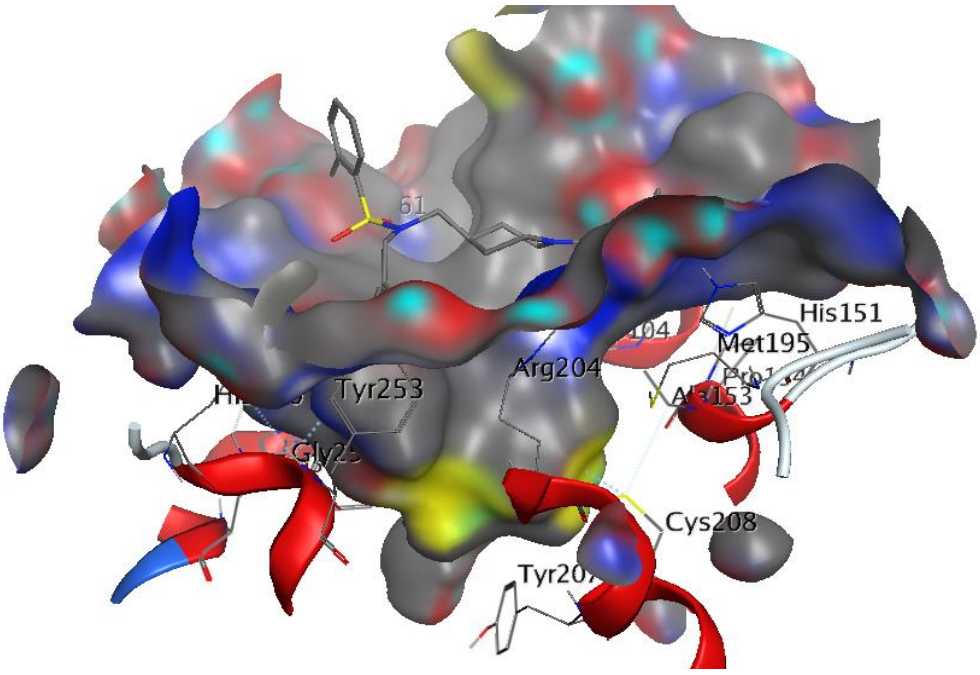


Figure 1: Binding site of PFT receptor (PDB ID: 3E37) in the complex with the substrate ethylenediamine ED5.

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III. RESULTS AND DISCUSSION

1. Developed QSAR model and validation

The **QSAR** analysis was performed using calculated molecular descriptors and the experimental values of the antimalarial activities for the thirty-six benzophenone derivatives, thirteen multiple linear regression (MLR) models based on the same size of training sets and using the 19 descriptors already selected after preprocessing on the basis of the correlation coefficient. It is interesting to observe that bpol, FMF and Alogp2 are the most important descriptors among the other models. In addition, it can be observed that **MLR** has the best model as compared to the other statistical parameters. Indeed, model 13 shows the best one between the 13 MLR models as expressed in the following **QSAR** equation 19 with 4 variables. The statistical parameters of all the generated models with threshold values were presented in Table S3.

$$\text{pIC50} = -6.818 - 6.234\ 10^{-2}\mathbf{ALogp2} + 0.156\ \mathbf{bpol} - 0.098\ \mathbf{MAXD} + 14.528\ \mathbf{FMF} \quad (18)$$

N = 27 $\mathbf{R^2}$ = 0.884; $\mathbf{R^2\ adj}$ = 0.865**; S** = 0.038**;** $\mathbf{N_{test}}$ **= 9; p** <0.0001**; F**= 42**;** $\mathbf{Q^2LOO}$=0.822

The obtained coefficient of correlation in equation (18) exhibits high value of correlation coefficient, 0.884, and low value of mean squared error, 0.038, which indicates that the model is more reliable. In addition, the coefficient p shows a lower value than 0.05 and the F-test has the value of 42, and these results demonstrate that the regression equation is statistically significant.

It can be observed the high adjusted value of the regression coefficient (R^2adj = 0.865) from the **QSAR** model, as shown in Table 3; In addition, it has the same value as that of the regression coefficient (R^2 =0.884), this indicates that the developed model has a perfect descriptive capacity for the descriptors and illustrates the real impact of descriptors used on **pIC50**.

Table 3: The validation parameters of the model which passed the three holds required for a QSAR model to be accepted

Validation Tools	Interpretation	Acceptable Value	model Value
$\mathbf{R^2}$	Co-efficient of determination	>0.6	0.884
$\mathbf{Q^2_{cv}}$	Cross-Validation Coefficient	>0.5	0.822
$\mathbf{R^2_{adj}}$	Adjusted R-squared	>0.6	0.865
press	predicted residual sum of squares	Press/ssy<	1.835
SSY	total sum of squares	0.4	0.209
VIF	Variance Inflation Factor	<5	1-2
$\mathbf{N_{Ext\ testset}}$	Minimum number of external and test sets	5	8.0
$\mathbf{R^2_{Test\ set}}$	Co-efficient of determination of external and test set	>0.5	0.821
$\mathbf{cR^2p}$	Coefficient of determination for Y-randomization	>0.5	0.811

Cross-validation is important way to explore the stability of a predictive model by using the analysis of the influence of each one of the individual objects that configure the final model.

The QSAR model expressed by equation (18) is cross-validated by its appreciable Q^2cv values (Q^2cv = 0.821) obtained using the leave-one-out method. The value of $\mathbf{Q^2cv}$ is higher than 0.5, which is important criterion for qualifying a **QSAR** model as valid [36].

In addition, the low PRESS/SSY ratio, 0.114, indicates the accuracy of the developed **QSAR** model used in this study and this is in agreement with the previous study, which states that the PRESS/SSY ratio should be lower than 0.4 [37]. The four model descriptors MAXDn, bpol, logp2 and FMF used in this study are shown in Table 4.

Table 4: Names of the model descriptors and their respective degree of contribution.

Java class Descriptor	Descriptor	Description	Class	ME%
ALOGP Descriptor	Alogp2	Square of ALogP	2D	0.06
BPolDescriptor	bpol	Sum of the absolute value of the difference between atomic polarizabilities of all bonded atoms in the molecule (including implicit hydrogens)	2D	45.38
Electrotopological State Atom Type Descriptor	MAXDn	Maximum negative intrinsic state difference in the molecule (related to the nucleophilicity of the molecule	2D	1.60
FMF Descriptor	FMF	Complexity of a molecule	2D	52.37

The simplest method of investigating occurrence of inter correlation is to calculate Pearson correlation coefficients for the descriptors in the model [38], reported in Table 5. The model's low correlation coefficients suggest that there is no substantial inter correlation between the descriptors. Furthermore, the multi-collinearity between the four descriptors for the model was detected by calculating their variation inflation factors (**VIF**), as shown in Table 5, using the following equation:

$$VIF_i = \frac{1}{1-R_i^2} \quad (19)$$

Where $\boldsymbol{R^2}$ is the value obtained by regressing and $\boldsymbol{i}$ is predictor on the other predictors. Surprisingly, the calculated **VIF** values with less than 2 were observed, and the results are tabulated in Table 5. This result confirms that there is no significant intercorrelation among the descriptors used in building the model, and this is in

agreement with a previous study, which states that the high **VIF** value more than 5.0 indicates that the model is unstable, while the value between 1.0 and 4.0 means that the model is acceptable [39].

Table 5: Correlation matrix (Pearson (n)) between the different descriptors and VIF values.

Descriptors	pbol	Logp2	FMF	MAXDn	VIF
pbol	1				2.018
Logp2	0.298	1			1.195
FMF	-0.181	-0.589	1		1.789
MAXDN	-0.103	0.028	0.140	1	1.034

Recently, the maximum negative intrinsic state difference (**MAXDn**), which relates with the molecular nucleophilicity have been studied as Kier-Hall intrinsic state atom type descriptor [40-41]. The ratio of Kier-Hall atomic electronegativity to the vertex degree is used to calculate atom. Thus, the number of bonds of the atom and encoding information were related to both partial charges of atoms and their topological negative relative to the whole molecule.

Since **MAXDn** has negative sign for the linear Eq. (18), increasing the value of descriptors via electrophilicity behavior of compounds has been shown to decrease the **pIC50** values.

FMF is a word that refers to the idea of molecular topology and the percentage of a molecular framework made up of terminal rings and a molecular bridge [42]. It has been shown that **FMF** correlates to the **ADMET** properties, such as solubility, permeability and Cytochrome P450 isoform 3A4 inhibition, as well [43]. As for the third descriptor **bpol** which is the sum of the absolute value of the difference between atomic polarizabilities of all bonded atoms in the molecule [44]. The results correlate with the antimalarial activities of the benzophenone derivatives. This implies that an overall increasing in the polarizability of the compound improves the antimalarial activity of benzophenone.

Molecular lipophilicity, usually quantified in log P, is an important molecular characteristic in medicinal chemistry and in rationalized drug design; the log P coefficient is well known as one of the main parameters for the estimation of lipophilicity of chemical compounds and determines their pharmacokinetic properties [45].

Log P has been linked to a wide range of biological activities, including pharmacological activity, toxicity, pesticidal action, genotoxic activity, and more. The lipophilicity is a main physico-chemical determinant influencing the bioavailability, and refers to the octanol/water partition coefficient descriptor **logP(o/w)** [46]. To study the lipophilicity of the benzophenone derivatives, the descriptor **logp2** was analyzed, and the results are presented in Table1. The negative coefficient of the **logp2** in model **MLR** suggests the increasing in the overall lipophilicity of the molecule that leads to decrease the **PFT** inhibitory activity of benzophenone derivatives.

On the other hand, the contribution of every descriptor in the built model was evaluated by the computation of the mean effect (**ME**) value [47] by using Eq. (20). The values for the **ME** are shown in both Figure 2 and Table 4:

$$ME_i = \frac{\beta_j \sum_{i=1}^{i=n} d_{ij}}{\sum_j^m \beta_j \sum_i^n d_{ij}} \qquad (20)$$

Where **Mei** is the mean effect of the descriptor ***j***, ***ßj*** represents the coefficient of the descriptor ***j,*** and ***dij*** represents the value of the selected descriptors of each compound and the total number of descriptors in the produced model is given by ***m***.

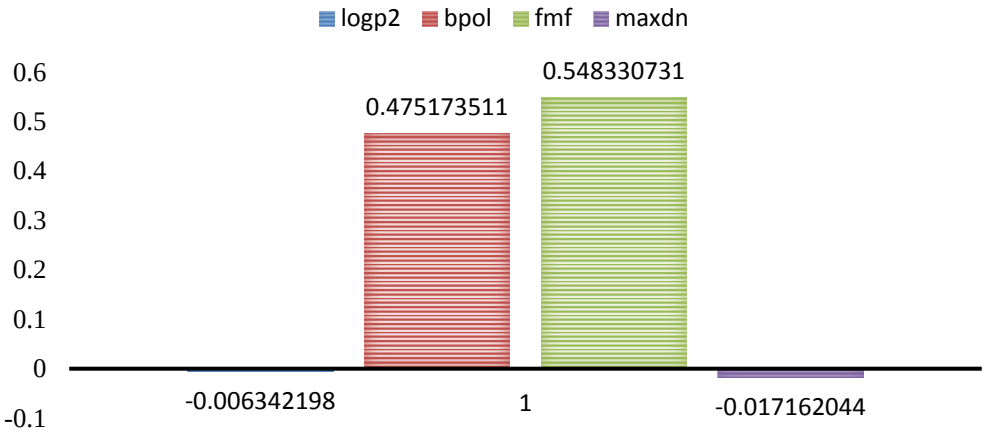


Figure 2: Contribution of descriptors in models.

Based on the calculated **ME** values, the greatest influence on the antimalarial activities among the four descriptors was **FMF**, and the trend is in the order of FMF > bpol > logp2 > MAXDn. This suggests that the **FMF** should be highly considered when designing high potent benzophenone derivatives.

Previously, Roy suggested the best way to estimate the true predictive power of a **QSAR** model is by comparing between the predicted and observed activities of an external test set of compounds in the developed **QSAR** model [48].

The values R^2pred = 0.821 indicate absolute quality of fitness the predicted model. The predicted activities of the test data compounds were studied for the **PFT** inhibitors by the developed **QSAR** model, and the results are tabulated in Table 1. Generally, Low residue values found in both the training set and the test, this indicated that the model has an ability to correlate activity and structure. The statistical analysis results showed that the correlation between experimental activity and predicted activity according to the model was highly significant.

The correlation between experimental and predicted values of **pIC50** of both the training and test sets obtained by **MLR** model is presented in Figure 3. The correlation showed clearly that the obtained **MLR** model from Eq. (18) represents reasonably well over the entire range of the **pIC50** values.

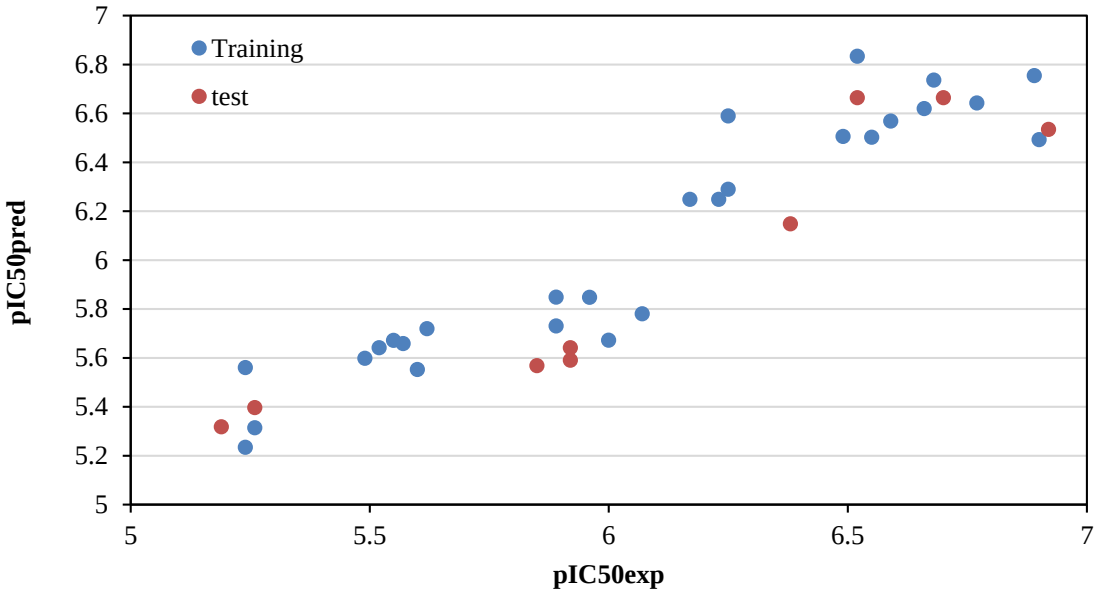


Figure 3: A correlation plot between predicted pIC50 (PRED) values on y-axis and pIC50 values on x-axis for both the training and test sets.

All the external validation results were above the threshold values for the various parameters presented in Table 3.

The squared correlation coefficient values between the observed and predicted values of the test set compounds (r^2) and (r^2_0), respectively were observed, and the model had satisfied the requirement of the term $(r^2 - r^2_0)/r^2$. This was in agreement with a previous study reported by Golbraikh and co-workers, which states that the value $(r^2 - r^2_0)/r^2$ exhibits less than 0.1.

In case of good external prediction, predicted values will be very close to observed activity values. Therefore, R^2 value will be very near to R^2_0 value. In the best case, r^2_m will be equal to r^2 whereas in the worst-case r^2_m value will be zero; including values of r^2_m<0.6 indicate these models are useless for external predictivity [49].

In the present study r^2_m value of the model [Equation 18] are acceptable (Table 6). This developed model passed all the Golbraikh and Tropsha criteria for the acceptability of the model (Table 7).

Table 6: Validation characteristics of developed model according to r^2m metrics and Concordance correlation coefficient

r_m^2parameter			Concordance correlation coefficient
r_m^2	$r_m'^2$	$\Delta r^2_{m(test)}$	CCC
0.553	0.533	0.02	0.946
>0.5	>0.5	<0.2	>0.85

Table 7: Golbraikh and Tropsha's criteria for the model.

R^2_{pred}	K	K'	$R^{\circ 2}$	$R'^{\circ 2}$	$\frac{R^2 - R^{\circ 2}}{R^2}$	$\frac{R^2 - R'^{\circ 2}}{R^2}$	$\lvert R_0^2 - R_{\circ}'^2 \rvert$
0.821	1,012	0.978	0.945	0.944	0.149	-0.148	0.001
>0.6	>0.85	<1.15	close to 1	close to 1	<0.1	<0.1	< 0.3

R: *Correlation coefficient between the predicted and observed activities*

$R^{\circ 2}$: *Coefficients of determination predicted versus observed activities*

$\boldsymbol{R'^{\circ 2}}$: *Coefficients of determination observed versus predicted activities.*

K and K': Slopes of the regression lines

2. Randomization test

The Y-Randomization method was carried out to validate the **MLR** and to detect and quantify chance correlations between the dependent variable and descriptors. By comparing the resulting scores between randomization test and the original **QSAR** equation which generated with non-randomized data, we have found that the new **QSAR** models, after several iterations, have low $\mathbf{R^2}$ and $\mathbf{Q^2_{LOO}}$ values, as shown in Table 8. This result indicates that the good **MLR** models obtained in this study cannot be attributed to the chance correlation of the training set.

Table 8: Y-randomization table for QSAR Analysis.

Model	**R**	**R^2**	**Q^2**
Original	0.940	0.884	0.822
Random 1	0.459	0.211	-2.191
Random 2	0.458	0.210	-0.233
Random 3	0.213	0.045	-0.437
Random 4	0.283	0.080	-5.857
Random 5	0.332	0.110	-0.779
Random 6	0.280	0.078	-0.426
Random 7	0.362	0.131	-1.084
Random 8	0.531	0.282	-0.440
Random 9	0.406	0.164	-3.358
Random 10	0.403	0.162	-0.573
Random Models Parameters			
Average r	0.373		
Average r^2	0.147		
Average Q^2	-1.538		
cRp^2	0.811		

3. Applicability Domain

The applicability domain (**AD**) approaches were used to estimate the prediction reliability for each modeled compound individually using many prediction methods. The plot of standardized residuals in prediction vs leverage values is a typical technique for visualizing the AD of a QSAR model. This plot, called as the Williams plot, allows for quick and easy graphical detection of response outliers and structurally influential chemicals in a model (hi > h*). Where h* is a threshold value, in fact, when a compound's leverage value is less than the crucial value h*, the chance of predicted and actual values agreeing is as great as it is for the training set chemicals. A high leverage chemical, on the other hand, is structurally distinct from the other chemicals and may thus be regarded outside the AD of the model. [50]. In this study, the results of the leverage values, standardized residuals of the observables and the test of the model which used to develop the applicability domain, are tabulated in Table 9. It has been observed that the standardized residual values for all compounds are in the range of −2.5 to 2.5. The lifts obtained are all below the critical value *h** = 0.55. Domain As shown in the developed Williams plot on the selected descriptors for predicting the PFT inhibitory activity all the compounds from the data set are in this area (Figure 4)

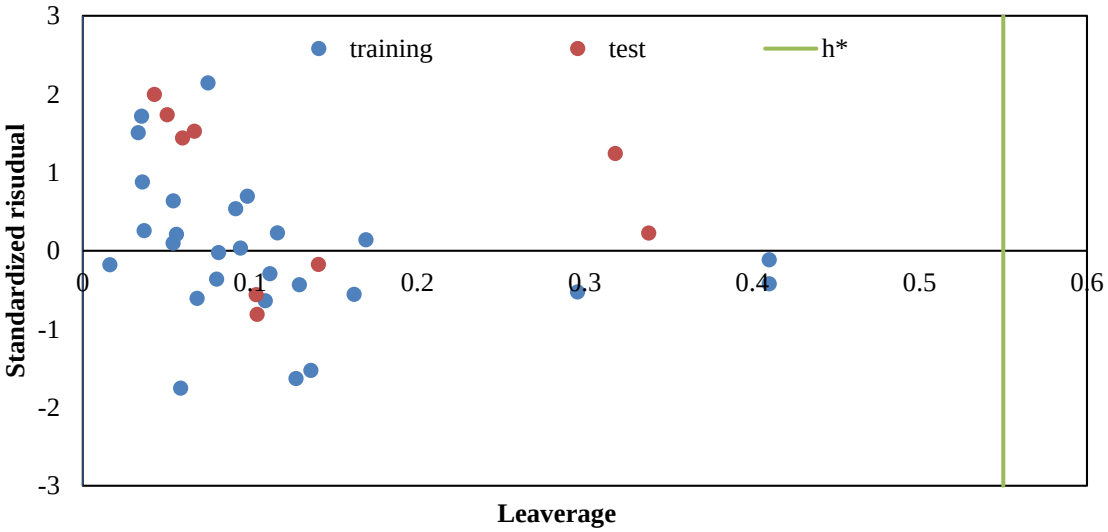


Figure 4: The Williams plot of MLR model for the training and test sets.

Table 9: The values of leverage and standardized residuals of the observables and test set

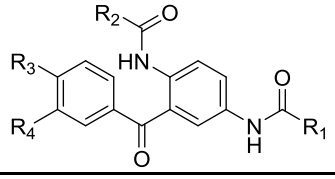
N. Compound	S. Residual	Leverage	N. Compound	S. Residual	Leverage
1	-1.528	0.136	**25**	0.033	0.094
2	0.877	0.035	**26**	0.097	0.054
4	-0.607	0.068	**27**	0.208	0.056
5	1.506	0.033	**28**	0.636	0.054
6	0.536	0.091	**29**	-0.180	0.016
7	0.227	0.116	**30**	0.256	0.036
8	0.139	0.169	**31**	-1.755	0.058
9	-0.557	0.162	**32**	-0.526	0.295

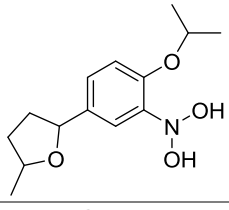
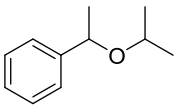
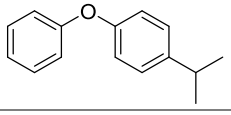
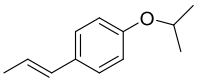
N. Compound	S. Residual	Leverage	N. Compound	S. Residual	Leverage
10	-0.638	0.109	**33**	-0.435	0.129
11	-0.362	0.079	**35**	-0.175	0.140
12	1.717	0.035	**36**	1.525	0.066
13	0.695	0.098	**3t**	-0.813	0.104
14	-1.631	0.127	**6t**	1.240	0.318
15	-0.293	0.111	**16t**	0.225	0.338
17	-0.024	0.081	**19t**	1.995	0.042
18	2.141	0.074	**20t**	-0.561	0.103
23	-0.116	0.410	**21t**	1.7364	0.050
24	-0.423	0.410	**22t**	1.4385	0.059

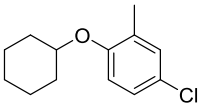
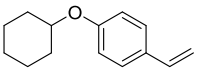
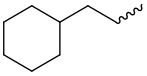
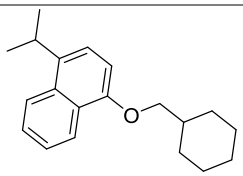
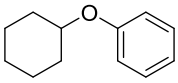
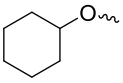
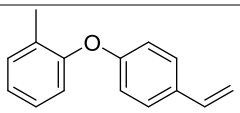
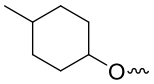
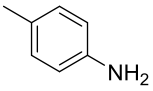
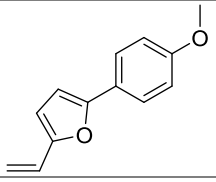
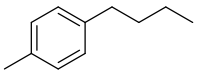
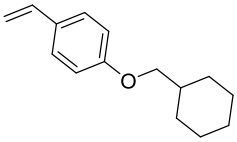
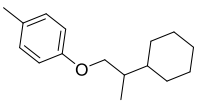
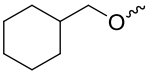
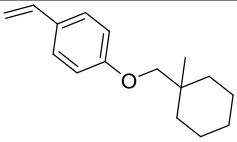
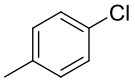
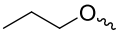
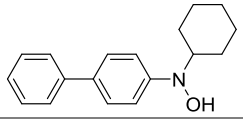
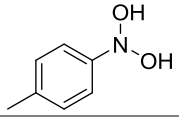
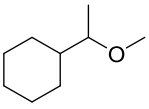
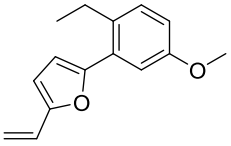
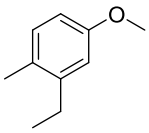
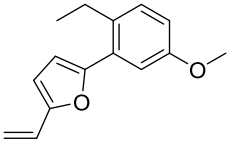
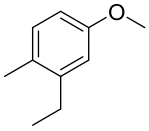
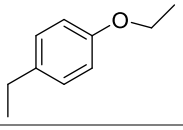
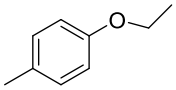
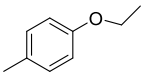
4. Design of novel derivatives

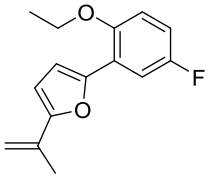
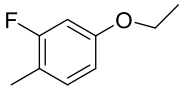
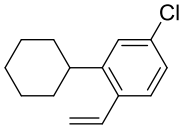
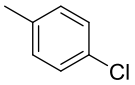
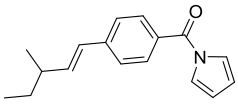
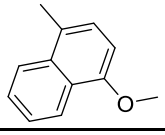
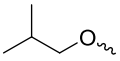
QSAR method has been playing important role for synthesis lead molecules and detect their biological activities. Therefore, the development of new benzophenones derivatives with strong affinity for **PFT** receptor could be achieved using core scaffolds that mimicked the best effective lead molecule. In this study, the equation of **MLR** model was applied for the 15 of newly designed benzophenone hybrids. A detailed profile of **bpol** and **FMF** descriptors was analyzed using the equation of **MLR** model, and the descriptors have been shown to increase the activity by increasing their values. Thus, the benzophenone backbones were developed by adding polar OH and NH_2 functionality and incorporation ring into the benzophenones scaffold. The predicted values and the calculated leverages (***h***) values were calculated for the different derivatives, and they are tabulated in Table 10.

Table 10: Structures of the newly designed inhibitors, predicted values and calculated h of pIC50 (in µM) used in this study.



COMP. N	R1	R2	R3	R4	pIC50 pred µM	*h*
P1				H	8.067	0.541
P2				H	7.783	0.391

P3				H	9.259	0.799
P4				H	10.374	0.866
P5				H	8.360	0.439
P6				H	7.647	0.279
P7				H	7.976	0.673
P8				H	7.842	0.360
P9				H	8.614	0.713
P10			$-OCH_3$	CH_3-	7.776	0.441
P11			$-CH_2CH_3$	$-OCH_3$	7.923	0.486
P12					8.804	0.509

P13				-F	7.588	0.399
P14				-F	8.250	0.434
P15				H	8.251	0.471

5. Molecular docking studies of the newly designed inhibitors

The Protein-Ligand interaction plays a vital role in structural based drug design [51-54]. In this present study, Molecular docking of benzophenone derivatives (**P1-P15**) was studied with **PFT** receptor, PDB ID: 3E37, to compare the binding interactions and the binding free energies (**ΔG**). The newly designed ligands with the most binding affinities and the synthetic substrate ethylenediamine inhibitor (**ED5**) used in this study are shown in Table 11. It can be observed that all the benzophenone derivatives occupy the same binding pocket, cavity 1, as that of synthetic substrate **ED5** (**Figure S1**).

The docking results of the complexes show that the binding free energies of benzophenone derivatives are in the same range as that of **ED5**, -10.28 kcal mol^{-1}, whereas **P1** has the lowest binding free energy of −12.38 kcal mol^{-1}. The complex **P7-PFT** show a binding energy of -8.79 kcal mol^{-1}, this indicates that **P7-PFT** have the lowest binding affinities.

In addition, the highest binding affinity, **P1-PFT** complex is engaged in hydrogen bonding interaction with the Asp354, Lys358, Cys301 and His250 amino acid residues, the hydrogen bonds were examined based on the acceptor-donor atom distance, as shown in Table 12. Upon comparing the occupancy of hydrogen bonds for the higher binding affinity **P1**, **22** and the ligand reference **ED5**, the results suggest that the newly designed complex **P1**-PFT has higher hydrogen bonds occupancy and provides stability to the PFT receptor. Furthermore, it was observed that benzophenone derivatives along with **P1** formed obvious hydrophobic interactions with Trp305 as shown in Figure 5.

Overall, the obtained results show that benzophenone derivatives can form stable complexes with PFT, and the best candidate **P1** binds to the receptor with higher affinity and stability.

Table 11: Binding free energies (in kcal mol^{-1}) of the PFT receptor interacting with the most active benzophenone derivatives and the design substrate.

Compound	Binding energy, ΔG	Compound	Binding energy, ΔG
ED5	-10.28	**P10**	**-8.796**
22	-9.06	**P11**	**-9.226**
P1	**-12.38**	**P12**	**-9.702**
P2	-9.372	**P13**	**-9.140**
P5	**-11.18**	**P14**	**-10.04**
P6	-9.490	**P15**	**-9.991**
P8	-9.129	/	/

Table 12: Molecular interactions between PFT receptor and P1, 22 and ED5

Compound	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
P1	O	ASP 354	H-donor	2.82	-6.6
	N	LYS 358	H-acceptor	3.95	-0.7
	O	CYS 301	H-acceptor	3.98	-0.7
	O	HIS 250	H-acceptor	3.49	-1.4
	6-ring	LYS 358	pi-cation	3.64	-3.0
22	N	HIS 250	H-acceptor	3.02	-2.8
	5- ring	TRP 305	H-pi	4.39	-1.1
ED5	N	CYS 208	H-donor	3.55	-1.1
	5-ring	TRP 305	pi-H	4.60	-0.7

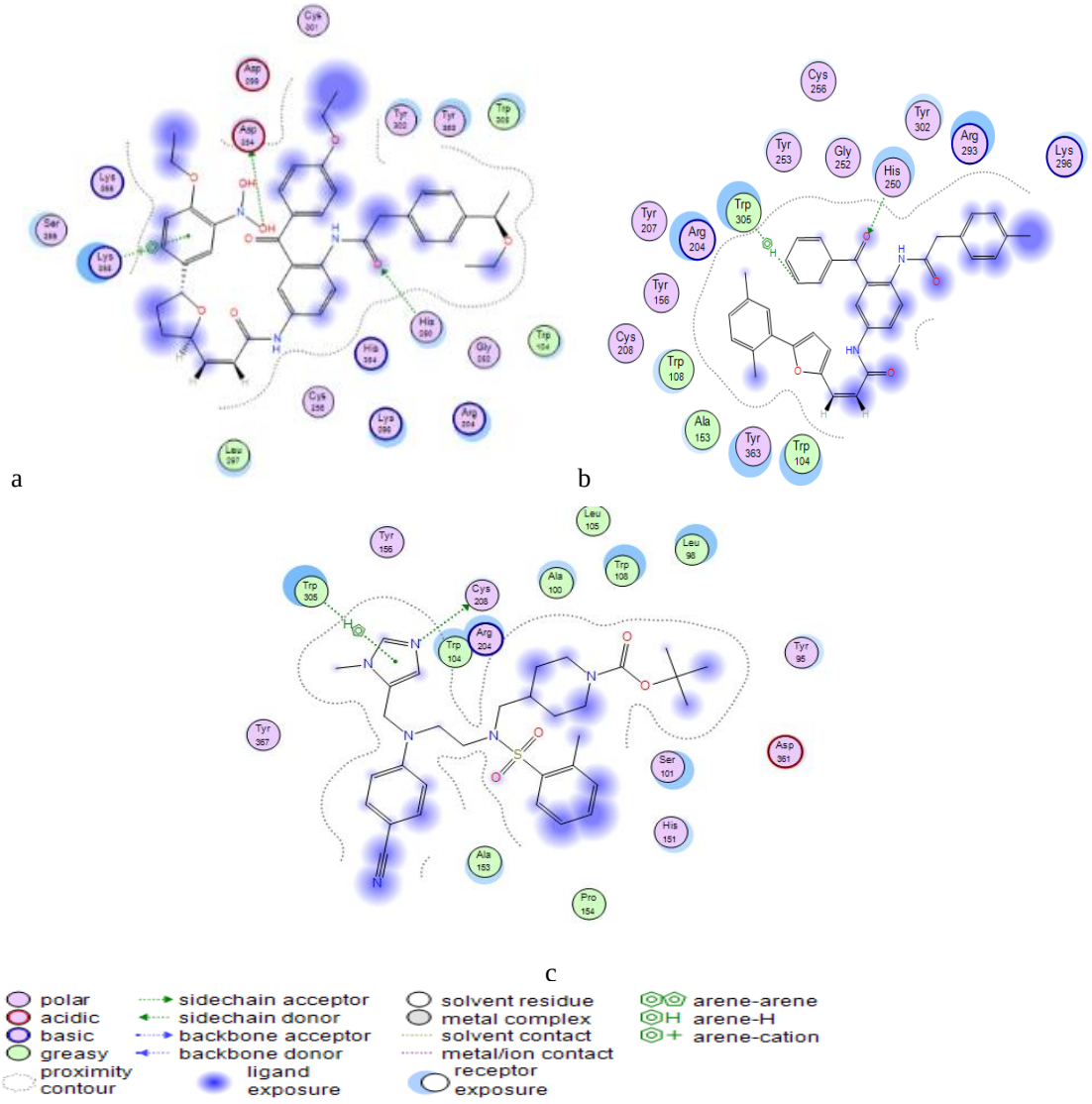


Figure 5: 2D representations of (a) Ligand ED5 (b) Ligand 22 (c) Ligand P1 docked in the binding pocket of the PFT. The important residues involved in the H-bond formation and hydrophobic interactions are highlighted.

CONCLUSION

Benzophenone derivatives as protein *farnesyltransferase* (PFT) inhibitors were modified using quantitative structure–activity relationship (QSAR) screening and molecular docking calculations. Thirty-six proposed ligands were studied by QSAR method, which used to propose and compare thirty MLR model results, the best model was found in agreement with the Tropsha and Golbraikh criteria. The results indicate that the descriptors such as pbol and FMF values modulate the activity of the molecules. In addition, the applicability domain (AD) results demonstrate that all the proposed compounds were within the defined domain. The pIC50 activity of fifteen proposed molecules was calculated using the proposed MLR model. Molecular docking results of the best predicted active compounds ($h < h^*$) with the PFT receptor demonstrate that **P1** binds well to the hydrophobic contacts and polar amino acid residues, leading to the increasing the binding affinity and thus enhancing the complex stability. Finally, the reasonable strategy for QSAR method and the high binding affinity for the newly designed ligands with PFT suggest that this benzophenone scaffolds may be worth exploring in the development of new inhibitors to treat malaria disease.

Acknowledgements

The authors are grateful to the University of Biskra, Algeria, and Middle East University; Amman, Jordan for the academic license of the software is used in this research article.

Supplementary Material

Table S1: Pearson's correlation matrix for descriptors used in QSAR model

	ALogP	ALogp2	AMR	Apol	Bpol	Lipo	MAXDP	DELS2	FMF	MAXDN	VABC	VAdjM	MW	AMW	WPATH	WPOL	XLogP	Zagreb	Tpsa
ALogP	1																		
ALogp2	0.002	1																	
AMR	-0.090	0.124	1																
Apol	0.120	0.367	0.724	1															
Bpol	-0.091	0.279	0.771	0.894	1														
Lipo	0.387	0.083	0.280	0.628	0.586	1													
MAXDP	0.000	0.252	0.785	0.882	0.836	0.552	1												
DELS2	0.133	0.182	0.380	0.380	0.360	0.105	0.380	1											
FMF	0.372	0.135	-0.68	-0.28	-0.58	-0.21	-0.460	-0.120	1										
MAXDN	0.177	-0.060	0.017	-0.08	0.013	0.193	-0.042	0.723	-0.12	1									
VABC	0.122	0.328	0.792	0.979	0.875	0.558	0.894	0.520	-0.33	0.033	1								
VAdj	0.187	0.359	0.647	0.944	0.794	0.536	0.828	0.617	-0.15	0.116	0.970	1							
MW	0.279	0.266	0.679	0.845	0.683	0.479	0.847	0.574	-0.13	0.121	0.890	0.895	1						
AMW	0.393	-0.158	-0.11	-0.21	-0.42	-0.18	-0.041	0.228	0.411	0.214	-0.124	-0.049	0.315	1					
WPATH	0.200	0.382	0.607	0.937	0.768	0.519	0.785	0.595	-0.09	0.086	0.957	0.994	0.874	-0.06	1				
WPOL	0.182	0.363	0.661	0.933	0.804	0.550	0.875	0.634	-0.20	0.152	0.959	0.982	0.905	-0.02	0.967	1			
XLogP	0.581	0.193	-0.02	0.335	0.232	0.743	0.132	-0.097	0.034	0.101	0.246	0.246	0.147	-0.24	0.263	0.243	1		
Zagreb	0.260	0.407	0.551	0.924	0.737	0.542	0.769	0.597	-0.01	0.107	0.932	0.985	0.876	-0.02	0.989	0.968	0.297	1	
Tpsa	-0.17	0.319	0.423	0.491	0.334	-0.27	0.368	0.537	0.014	-0.065	0.565	0.620	0.482	-0.02	0.641	0.579	-0.311	0.607	1

Table S2: List of physiochemical descriptors used for the best model

Descripteurs	Description
ALogP	Ghose-CrippenLogKow
ALogP2	Square of ALogP
Bpol	Sum of the absolute value of the difference between atomic polarizabilities of all bonded atoms in the molecule (including implicit hydrogens)
TPSA	Topological polar surface area
MAXDN	Maximum negative intrinsic state difference in the molecule (related to the nucleophilicity of the molecule). Using deltaV = (Zv-maxBondedHydrogens) /(atomicNumber-Zv-1). Gramatica, P., Corradi, M., and Consonni, V. (2000). Modelling and prediction of soil sorption coefficients of non-ionic organic pesticides by molecular descriptors. Chemosphere 41, 763-777.
MAXDP	Maximum positive intrinsic state difference in the molecule (related to the electrophilicity of the molecule). Using deltaV = (Zv-maxBondedHydrogens) /(atomicNumber-Zv-1). Gramatica, P., Corradi, M., and Consonni, V. (2000). Modelling and prediction of soil sorption coefficients of non-ionic organic pesticides by molecular descriptors. Chemosphere 41, 763-777.

Descripteurs	Description
FMF	Complexity of a molecule
MW	Molecular weight
AMW	Average molecular weight (Molecular weight / Total number of atoms)
WPOL	Weiner polaritynumber
WPATH	Weiner path number
XLogP	Partition coefficient of Wang's octanol water
Lipo	Lipoaffinity index
DELS	Sum of all atoms intrinsic state differences (measure of total charge transfer in the molecule). Using deltaV = (Zv-maxBondedHydrogens) /(atomicNumber-Zv-1). Gramatica, P., Corradi, M., and Consonni, V. (2000). Modelling and prediction of soil sorption coefficients of non-ionic organic pesticides by molecular descriptors. Chemosphere 41, 763-777.
AMR	Molarrefractivity
Apol	Sum of the atomic polarizabilities (including implicit hydrogens)
vabc	Van der Waals volume calculated using the method proposed in [Zhao, Yuan H. and Abraham, Michael H. and Zissimos, Andreas M., Fast Calculation of van der Waals Volume as a Sum of Atomic and Bond Contributions and Its Application to Drug Compounds, The Journal of Organic Chemistry, 2003, 68:7368-7373]
V adjamat	Vertex adjacency information (magnitude)
Z agreb	Sum of the squares of atom degree over all heavy atoms i

Table S3: Statistical results of different QSAR model.

	Descriptor	R^2 *adj*	S	R^2 *test*
1	*WPATH-TopoPSA*	0.686	0.099	0.358
2	*bpol FMF*	0.640	0.175	0.645
3	*bpolFMF*	0.679	0.133	0.530
4	*WPATH-XLogP-TopoPSA*	0.649	0.119	0.434
5	*bpol-FMF-WPATH*	0.797	0.082	0.408
6	*ALogp2bpolFMF*	0.669	0.117	0.664
7	*ALogp2-bpol-FMF-MW*	0.707	0.103	0.578
8	*ALogp^2 bpol-FMF-TopoPSA*	0.755	0.135	0.508
9	*bpol- MW-WPATH*	0.823	0.081	0.395
10	*bpol-MAXDN-FMF-XLogP-TopoPSA*	0.798	0.092	0.477
11	*Apol-MAXDN-FMF-MW-AMW*	0.708	0.082	0.026
12	*ALogp2-bpol-MAXDN-FMF-TopoPSA*	0.758	0.082	0.555
13	*ALogp2-bpol-MAXDN-FMF*	0.865	0.038	0.821

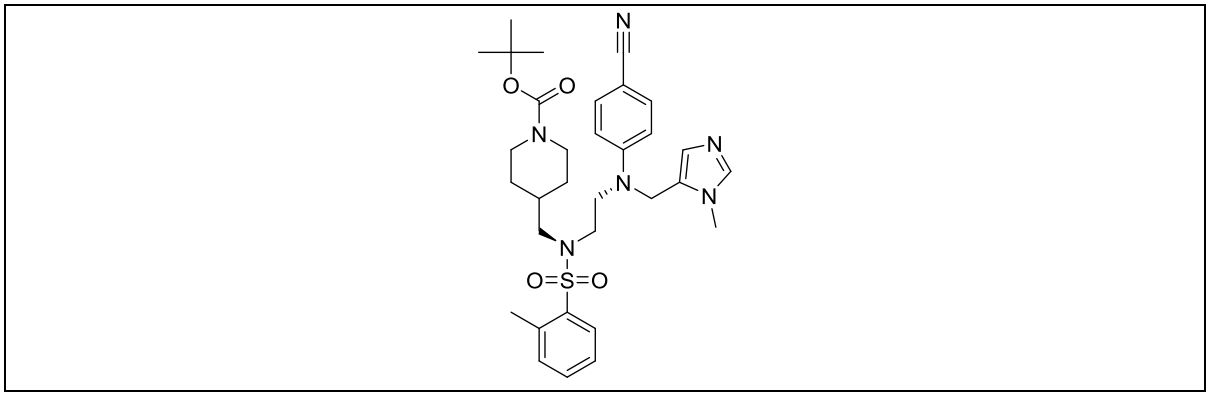


Figure S1: structure of ligand of reference tert-butyl 4-({(2-{(4-cyanophenyl) (1-methyl-1H-imidazol-5-yl)methyl]amino}ethyl)[(2-methylphenyl)sulfonyl]amino}methyl)piperidine-1-carboxylate C_{32} H_{42} N_6 O_4 S

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تحقيقات التركيب الكمي للنشاط (QSAR) وتحليل الالتحام الجزيئي لمثبطات بروتين البلازموديوم فرنازيل تراتسفيراز كعوامل قوية مضادة للملاريا

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ملخص

يعتبر تطوير مثبطات farnesyltransferase على أساس سقالة بنزوفينون الموجهة ضد falciparum Plasmodium استراتيجية في علاج الملاريا. في هذا العمل، تم إجراء علاقة التركيب الكمي بالنشاط (QSAR) للتنبؤ بالأنشطة المثبطة للبروتين (PFT) farnesyltransferase لسلسلة من 36 مشتقًا من مشتقات بنزوفينون. تم تقسيم مجموعة البيانات إلى مجموعتين فرعيتين من مجموعات التدريب والاختبار، وأفضل نموذج باستخدام الانحدار الخطي المتعدد (MLR)، مع قيم الصلاحية الداخلية والخارجية $R^2 = 0.884$، $R^2adj = 0.865$، $R^2pred = 0.821$، $R^2p =$ $Q^2cv = 0.822$ و0.811 بالاتفاق مع معايير Tropshaو Golbraikh.تم تحديد مجال التطبيق (AD) باستخدام مخطط ويليامز لوصف الفضاء الكيميائي للنموذج المستخدم في هذه الدراسة. يوضح النموذج أن الأنشطة المضادة للملاريا للبنزوفينون تعتمد على واصفات logP وbpol وMAXDn وFMF. دفعتنا هذه المؤشرات إلى تصميم مثبطات جديدة للبنزوفينونات PFT والتنبؤ بقيمة أنشطتها المضادة للملاريا بناءً على معادلة MLR. تكشف نتائج الإرساء أن البنزوفينونات المصممة حديثًا ترتبط بالجيب الكارهة للماء والتلامس القطبي مع التقارب العالي. يمكن أن تساعد النتائج المتوقعة من هذه الدراسة في تصميم بنزوفينونات جديدة كمثبطات لـ PFT البشري مع أنشطة مضادة للملاريا عالية.

الكلمات الدالة: QSAR، الالتحام، بنزوفينون، مثبط PFT، مضاد للملاريا.

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تاريخ استلام البحث 2021/9/17 وتاريخ قبوله للنشر 2022/1/12.

Jordan Journal of Pharmaceutical Sciences, Volume 15, No. 3, 2022

Chemical Constitution, *In-silico* Molecular Docking Studies and Antibacterial Activity of Flower Essential Oil of *Artabotrys hexapetalus*

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ABSTRACT

The isolation of the volatile constituents from the flowers of *Artabotrys hexapetalus* was carried out using a simple headspace solvent-trapping technique and identified by GC-MS analysis. The major compounds are ethyl acetate 53.6%, isobutyl acetate (29.4%) and ethyl benzoate (14.2%). The odour of the solution obtained from this method was found to be similar to that of the fresh flowers. Further the essential oil from *A. hexapetalus* was obtained for the first time from India by hydro distillation using a Clevenger type apparatus and analysed by GC-MS. The plant yielded 1.26%, of the essential oils from the flower. The analysis lead to the identification of 28 compounds representing 96.17% of the total oil. The essential oil consists of predominantly oxygenated sesquiterpenes (51.91%) followed by sesquiterpenes (43.31%) and small quantities of monoterpenes (1.24%) and other compounds (1.34%). The main constituents of the essential oil obtained from the flowers of *A.hexapetalus* are β-caryophyllene (18.69%), caryophyllene oxide (14.54%), cubenol (12.53%) and ledol (11.5%). The essential oil showed antibacterial activity against bacterial strains *Streptococcus pneumonia*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* exhibiting a zone of inhibition of 16.4, 15.7, 17.5 and 14.5 mm and MIC value of 2.5, 5.0, 2.5, 5.0 mg/ml respectively. Molecular docking analysis indicated that the essential oil constituents are nucleic acid and cell wall synthesis inhibitors. So it is worth to include this in cosmetics and fragrances.

Keywords: *Artabotrys hexapetalus*, essential oil, GC-MS, sesquiterpenes, β-caryophyllene, antibacterial, docking.

1. INTRODUCTION

Artabotrys species are traditionally used for a wide range of diseases like cholera, scrofula and malaria. The fruits and leaves of *Artabotrys* species are utilized as animal feeds, predominantly for goats, chimpanzees and cattle [1]. Due to the fragrance of the flowers of *Artabotrys* species, they are used as flavouring agents, in the manufacture of perfumes and for making stimulating tea-like beverages. Boiled juice of flowers is a stimulating beverage and used to treat vomiting, biliousness, blood diseases, heart and bladder disorders, itching and leucoderma[2]. They are used in the treatment of bad breath, headache, sweating, and thirst and also used as cardiotonic[3,4]. *A. hehapetalus* has numerous activities such as antispermatogenic, antiandrogenic, antioxidant, antimicrobial, and antidenaturation of protein, antiproteinase and anti-inflammatory.

The flowers from *A. hexapetalus* (Fig 1) have a sweet and fresh odour and however it was investigated only once from Thailand[5] to identify the volatile compounds responsible for its odour and from Vietnam to study the chemical composition of the essential oil[6]. To our knowledge we are investigating for the first time to identify these compounds from India. The sweet and fresh smell from this flower comes only between 5 to 8 a.m. in the morning and 6 to 8 p.m. in the evening[5]. It means that the compounds responsible for this odour from the flower are

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Received: 9/10/2020 Accepted: 20/1/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.408*

released only within this period of time. Therefore, it is important that the onsite sampling and preconcentration steps are to be focussed in order to identify the volatile constituents of the flowers from *A. hexapetalus*. Thus the objective of the present work is to identify the chemical composition of the essential oil from flowers of *A. hexapetalus* after giving due importance to the onsite sampling and precondition step. Further another objective of the present work is to investigate the antimicrobial activity of the essential oil obtained from *A. hexapetalus* and to find a mechanism of the action of antimicrobial activity by molecular docking study. Several drugs that are currently available to the public for the treatment of different diseases have been developed based on in silico approaches. For example, Zanamivir, used to treat influenza, was developed using computer-assisted design[7] [A]. Nelfinavir and Saquinavar are used in the treatment of HIV and were also developed by computational methods[8]. [B].

Fig.1. Flower from *A. hexapetalus*

2. Materials and Methods

2.1. Plant Material

The flower of *A. hexapetalus* were collected (200 g) from the Coimbatore District (coordinates: 10.9880° N, 76.7740° E), Tamilnadu, India between 6 to 7 a.m. in the morning during the month of January 2018. The plant material was authenticated by Dr. R. Gopalan, Professor of Botany Department, KAHE, Coimbatore (Voucher No. KAHE/CHE/2018/102).

2.2. Extraction of Essential Oil

After the onsite collection of the flowers between 6 to 7 a.m. in the morning the components were extracted immediately using a simple head space-solvent technique. In this method about 500 g of the flower were taken in an Erlenmeyer flask (500 ml capacity) fitted with a one holed rubber cork. Using an aquarium pump fresh air was blown in continuously through the inlet of the flask for nine hours. The vapour collected on the top surface of the flask was allowed to pass in to a round bottomed flask having 30 ml of methylene chloride solvent. This was repeated four times and the combined resulting solution was concentrated to 2 ml in a rotary evaporator and the concentrate was analysed by GC-MS.

Fresh flowers obtained (500 g) were chopped into small pieces and subjected to hydro distillation. A quantity of 60 g of the flowers *A. hexapetalus* was added to 300 ml of distilled water in a one litre flask fitted with a Clevenger apparatus and a condenser through which cold water was circulated to ensure condensation of essential oils for 2 h. This was repeated twice. At the end of the distillation, two phases were observed, an organic phase (essential oil) and an aqueous phase (aromatic water). The essential oil was collected, dried under anhydrous sodium sulphate. Until further analysis the resulted oil was stored at 4°C in a refrigerator.

2.3. Determination of Chemical Composition of Essential Oil

GC-MS along with an ESI system with the ionization energy of 70 eV was utilized for essential oil composition

analysis. Agilent Technologies, 7890A, with a HP-5MS column (5 % phenyl methylpolysiloxane) 30 m × 0.25 mm ID × 0.25 μm film. The mass spectrometer with an ion-trap analyzer was set at 1508 for all analyses with an electron multiplier voltage of 1058V. Scanning was performed from m/z 39 to 400 in 70 eV EI (electronic impact) at 1 scan/ s-1 and the selected split ratio was 1:10. Helium (99.99%) with the flow rate of 1ml/min was used as the carrier gas. The injection part of the instrument was set at a temperature of 250ºC. The initial temperature of the column was maintained at 40°C for 1min, and then gradually increased to 240ºC at the rate of 30ºC/min. Essential oil constituents were tentatively identified by comparison of their GC retention indices (RI), determined with reference to a homologous series of C8-C20 n-alkanes and with those of available authentic standards and literature. Confirmation of such identification was done by comparing their mass spectral fragmentation patterns with those stored in the MS database (NIST 2005 and Wiley 7N libraries) and with mass spectra literature data. Components relative concentrations were obtained with the response factors to the FID.

2.4. Antibacterial screening

The antibacterial screening by zone of inhibition method and determination of Minimum inhibitory concentration (MIC) were determined using the bacterial strains like *Streptococcus pneumonia*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* by the method as we reported earlier[9].

2.5. Molecular Docking

2.5.1. Preparation of Proteins and ligands

The three-dimensional structure of the proteins with PDB id: 3UDI, 3TYE, 3TTZ and 1JZQ were downloaded from the RCSB protein Data Bank and saved in PDB file format, for further studies in Auto dock vina under PyRx 0.8 Platform.

The compounds present in the essential oil obtained from *A. hexapetalus* were selected for docking studies. Molecular docking study has been carried out using the PyRx Version 0.8 docking program. Ligands 2D structures were drawn and converted into 3D using Chem Office 2002. After energy minimization of the ligands, it was docked with the protein's target sites (amino acids). Discovery studio was used to convert 2D in to 3D structure and the energy was minimized using AM1 method. To minimise the energy to minimum RMS gradient of 0.100 was set in each interaction. All structures were saved as PDB file format. All the ligand structures were then saved in SDF file format, to carry out docking in Autodock vina[10]. A grid box with dimension of 40 x 40 x 40A with 0.37A spacing and cantered on 29.47, 47.99, 8.86 was created around the binding site on proteins. The centre of the box was set a ligand centre, and grid energy calculations were carried out.

3. Results and Discussion

The isolation of the volatile constituents from the flowers of *A. hexapetalus* was carried out using a simple headspace solvent-trapping technique and the headspace vapour was flushed with air and collected in solvent methylene chloride. When analysed by GC-MS, Six compounds were identified from the resultant concentrated methylene chloride solution. The identified volatile compounds are ethyl acetate 53.6%, isobutyl acetate (29.4%) and ethyl benzoate (14.2%) as major compounds and ethyl propionate (1.6%), ethyl octonate (0.7%) and isobutyl valerate (0.43%) as minor compounds. The odour of the solution obtained from this method was identified to be similar to that of the fresh flowers. The presence of ethyl benzoate and ethyl propionate in the present investigation of the volatile constituents from the flowers of *A. hexapetalus* make the smell of the flowers of *A. hexapetalus* from India different from the flowers of *A. hexapetalus* from Thailand[5].

The chromatogram obtained from the GC-MS analyses was shown in Figure 2. It resulted in the identification of 28 compounds (Figure 3) representing 96.17% of the oil. The plant yielded 0.36%, (0.68 g) of the essential oils from the flower (the average yield of three

distillations). The essential oil consists of predominantly oxygenated sesquiterpenes (51.91%) followed by sesquiterpenes (43.31%), monoterpenes (1.24%) and small quantities of other compounds (1.82%). The GC-MS analysis results are summarized in the Table 1. Caryophyllene oxide (14.54%), β-caryophyllene (18.69%), cubenol (12.53%) and ledol (11.5%) were the main constituents of the essential oil of the flowers. It is having a strong green odour and differs a lot from the smell of the fresh flowers. This is due to the reason that in the high temperature prevailed during the hydrodistillation, the enzymatic processes that were responsible for the odour formation and release of the compounds would have denatured[11]. Hence the compounds which contribute to the significance odour could not be accumulated in the obtained essential oil.

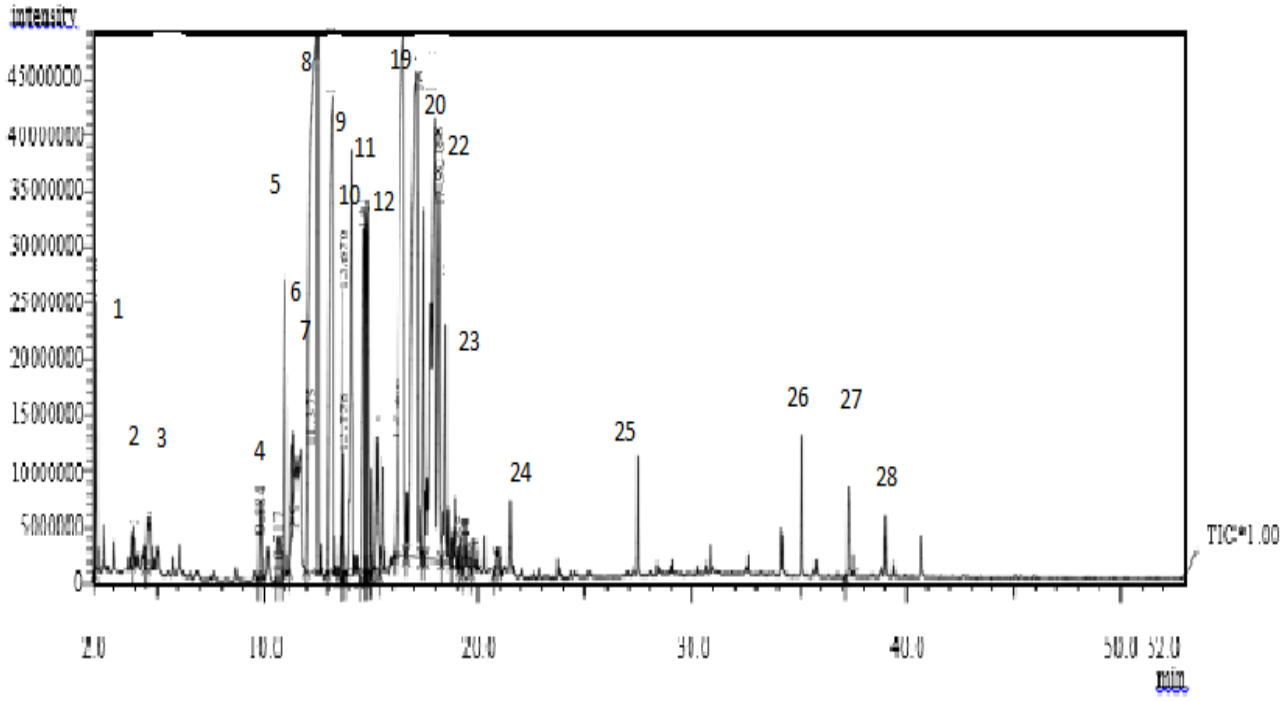

Figure 2: GC-MS Chromatogram of essential oil obtained from *A. hexapetalus*.

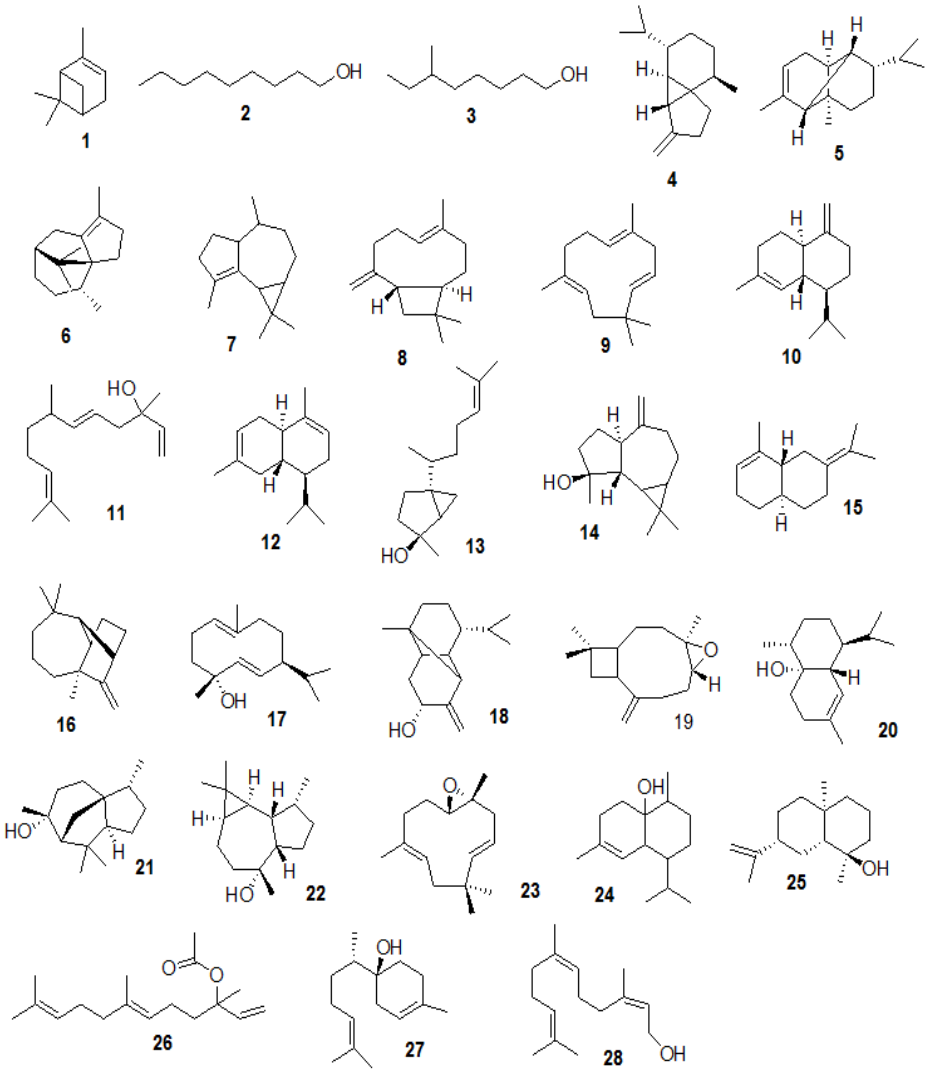

Fig. 3 Structure of the compounds identified from the essential oil from the flowers of *A. hexapetalus*

In an earlier study twenty-eight components comprising of sesquiterpenes hydrocarbons (33% of the oil) and oxygenated sesquiterpenes (47.7%) were reported from the flower oil of *A. hexapetalus* collected from Vietnam. The major compounds are α-copaene (8.1%), β-elemene (1.0%), β-caryophyllene (11.4%), α-humulene (3.5%), γ-muurolene (3.5%), caryophyllene oxide (31.5%), and humulene epoxide (10.01%) [12].

Table 1. Essential oil composition of *Artabotrys hexapetalus* as determined by GC-MS

Compound.No	Retention time[a]	Compound[b,c]	%[d]	Molecules formulae	Retention Index[e]
1	2.1	α-pinene	1.24	$C_{10}H_{16}$	934
2	3.94	1-Nonanol	0.20	$C_9H_{20}O$	1089
3	4.68	6-Methyloctan-1-ol	0.64	$C_9H_{20}O$	1109
4	10.71	β-Cubeben	0.41	$C_{15}H_{24}$	1333
5	11.013	Copaene	3.91	$C_{15}H_{26}$	1375
6	11.375	cyperene	1.00	$C_{15}H_{24}$	1398
7	11.452	α -Gurjunene	0.59	$C_{15}H_{24}$	1405
8	12.53	β-Caryophyllene	18.67	$C_{15}H_{24}$	1420
9	13.263	Humulene	8.24	$C_{15}H_{24}$	1449
10	13.670	γ-cadinene	1.97	$C_{15}H_{24}$	1505
11	13.778	Nerolidol	0.60	$C_{15}H_{26}O$	1520
12	14.19	β-cadinene	4.88	$C_{15}H_{24}$	1530
13	14.70	Sesquisabinene Hydrate	3.86	$C_{15}H_{26}O$	1534
14	14.79	Spathulanol	2.29	$C_{15}H_{26}O$	1566
15	14.97	Selina-3,7 (11)-diene	3.18	$C_{15}H_{24}$	1567
16	14.98	Longifolene	0.21	$C_{15}H_{24}$	1568
17	15.067	Germacrene D-4-ol	0.46	$C_{15}H_{26}O$	1569
18	15.29	β-Copaene-4α-ol	1.16	$C_{15}H_{26}O$	1570
19	16.58	Carryophyllene oxide	13.46	$C_{15}H_{26}O$	1573
20	17.18	Cubenol	12.53	$C_{15}H_{26}O$	1590
21	17.52	cedrol	2.49	$C_{15}H_{26}O$	1592
22	18.14	Ledol	11.57	$C_{15}H_{26}O$	1594
23	18.51	Humulene epoxide	2.18	$C_{15}H_{26}O$	1597
24	21.42	1-Cubenol, epi	0.58	$C_{15}H_{26}O$	1614
25	27.12	Selin-11-en-4α-ol	0.25	$C_{15}H_{24}$	1641
26	35.04	Nerolidol-Epoxyacetate	0.50	$C_{17}H_{28}O_2$	1687
27	37.21	β-Bisabolol	0.41	$C_{15}H_{26}O$	1689
28	39.06	Farnesol	0.32	$C_{15}H_{26}O$	1733

[a]Compounds are listed in order of their elution from a HP-5MS column.

[b]Identification: MS, based on comparison with NIST 14 MS databases;

[c]Retention index from NIST 14 and Wiley 275 mass spectral databases.

[d]Quantification was done by external standard method using calibration curves generated by running GC analysis of representative authentic components

[e]Retention index on the HP-5MS column, calculated using homologous series of C_9–C_{18} alkanes.

From Thailand the essential oil was obtained by four different process like simple headspace solvent-trapping technique, solvent extraction, hydro distillation, and solid phase micro extraction (SPME) and the identified compounds were reported[5]. Oil from the hydro distillation method showed the presence of thirty one components, of which the major components were β-gurjunene (30.0%), Globulol (13.8%) and β-caryophyllene (10.1%). Essential oil obtained from the same source by solvent extraction led to the identification of thirty one components of which the major compounds were isopentyl acetate (12.6%), linalool (7.7%), 2-methylbutyl acetate (7.7%), limonene (5.7%) and 3-methylbutanol (5.7%). Alternatively when it was performed with solid-phase micro extraction (SPME) methods, thirty nine components were identified with ethyl acetate (12.8%) and isobutyl acetate (39.5%) as the major components[5]. Further Cadinol, spathulenol, β-caryophyllene oxide and cubenol (-) were reported from the essential oil obtained from Tanzania. The volatile constituents are dominated by sesquiterpene hydrocarbons and oxygenated sesquiterpenoids[14].

The present study showed that the chemical constituents from the essential obtained from Indian *A. hexapetalus* are found to be different with the essential oil obtained by the hydro distillation method from Tanzania[13] and Vietnam[12] and Thailand. In our study the quantity of β-caryophyllene is more than the caryophyllene oxide where as in the above studies caryophyllene oxide is more than that of β-caryophyllene. The study indicated that the sesquiterpenes β-caryophyllene oxide to be present in almost all essential oils obtained by hydro distillation of the *A. hexapetalus*. β-caryophyllene oxide was reported to exhibit mosquito repellent activity.

3.1. Antibacterial Screening

The diameter of zone of inhibition was measured in mm and presented in the table 2. The essential oil from *A. hexapetalus* exhibited inhibitory activity against all the bacterial strains *Streptococcus pneumonia, Staphylococcus aureus, Streptococcus pyogenes* and *Pseudomonas aeruginosa* with MIC values of 2.5, 5.0, 2.5, 5.0 mg/ml and narrow inhibition zones of 16.4, 15.7, 17.5 and 14.5 mm respectively. Ampicillin was used as a positive control. Overall the results suggest that the essential oil of *A. hexapetalus* have a potential antibacterial activity. The activity is attributed to the various constituents present in the essential oil obtained from the flowers of *A. hexapetalus.*

3.2. Molecular Docking

Antibiotics may either kill or inhibit the growth of bacteria by different mechanisms[15,16]. Now, in the current study, the knowledge on the target proteins of currently used antibiotics[17,18] is extended to the phytoconstituents which is identified from *A. hexapetalus* in order to examine their affinity with the bacterial proteins that are well known targets for some antibiotics with different mechanism of action such as cell wall synthesis, inhibitors of nucleic acid synthesis and antimetabolites. In the present study we carriedout the molecular docking studies with 3UDI (acinetobacter baumannii in complex with penicillin G), 3TYE (dihydropteroate synthase), 3TTZ (DNA gyrase) and 1JZQ (Isoleucyl-tRNA synthetase) proteins which represent the above three mechanisms. The docking score of the ligands with the protein 3TTZ and 1JZQ are not encourageable and hence not pursued further.

One of the target protein (PDB id: 3UDI) is from murD ligase which is involved in the cell wall synthesis and the other target is dihydropteroate synthase enzyme (DHPS; PDB id: 3TYE) a key component in the folate pathway of bacteria and primitive eukaryotes. The essential oil constituents were docked against these two targets and the compounds with a reasonable docking score (Kcal/mole) are presented in the table 3. Most of the ligands exhibited hydrophobic interactions (Figure 4a-4d) with the target proteins which are evidenced by their docking scores. This indicates that the essential oil components of *A. hexapetalus* behave as inhibitors of nucleic acids and cell wall synthesis inhibitors which involve in cell well synthesis. So we hypothesise that these essential oil constituents first interact with the cell wall to destruct the cell structure and then inhibits the normal synthesis of DNA that are

required for bacterial growth. β-Lactams act entirely outside the cell membrane, in the final phase of peptidoglycan biosynthesis. Sulfonamides inhibit the action of dihydropteroate synthetase (with p-aminobenzoic acid (PABA) as substrate), preventing the synthesis of dihydrofolic acid[17,20]. So, from the present study we can say that these compounds act on the multi targets and may serve as antibacterial agents.

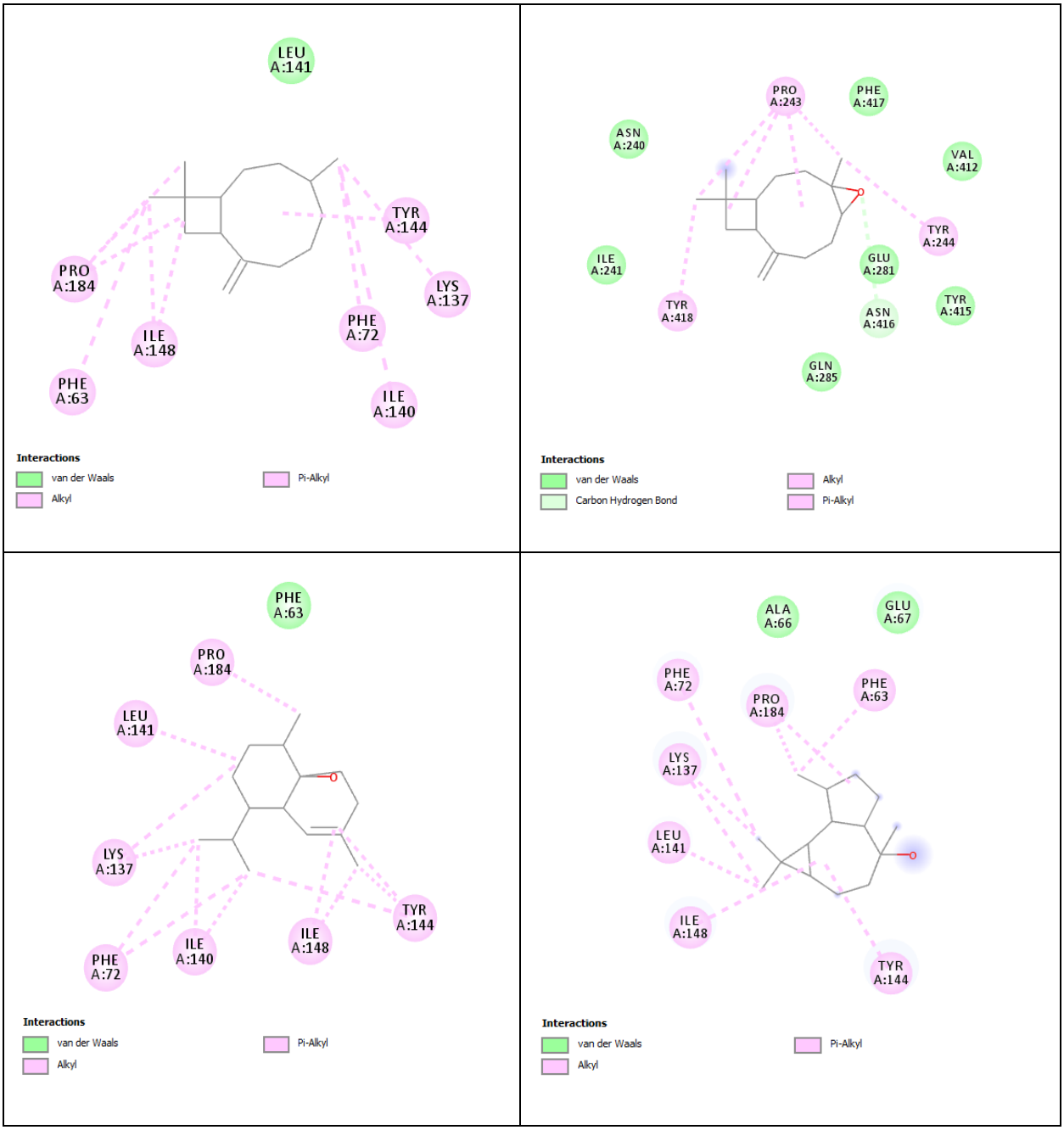

Figure 4a: Molecular docking of Trans (beta)-caryophyllene, caryophyllene oxide, Cubenol and Ledol against the target protein with the PDB id: 3UDI

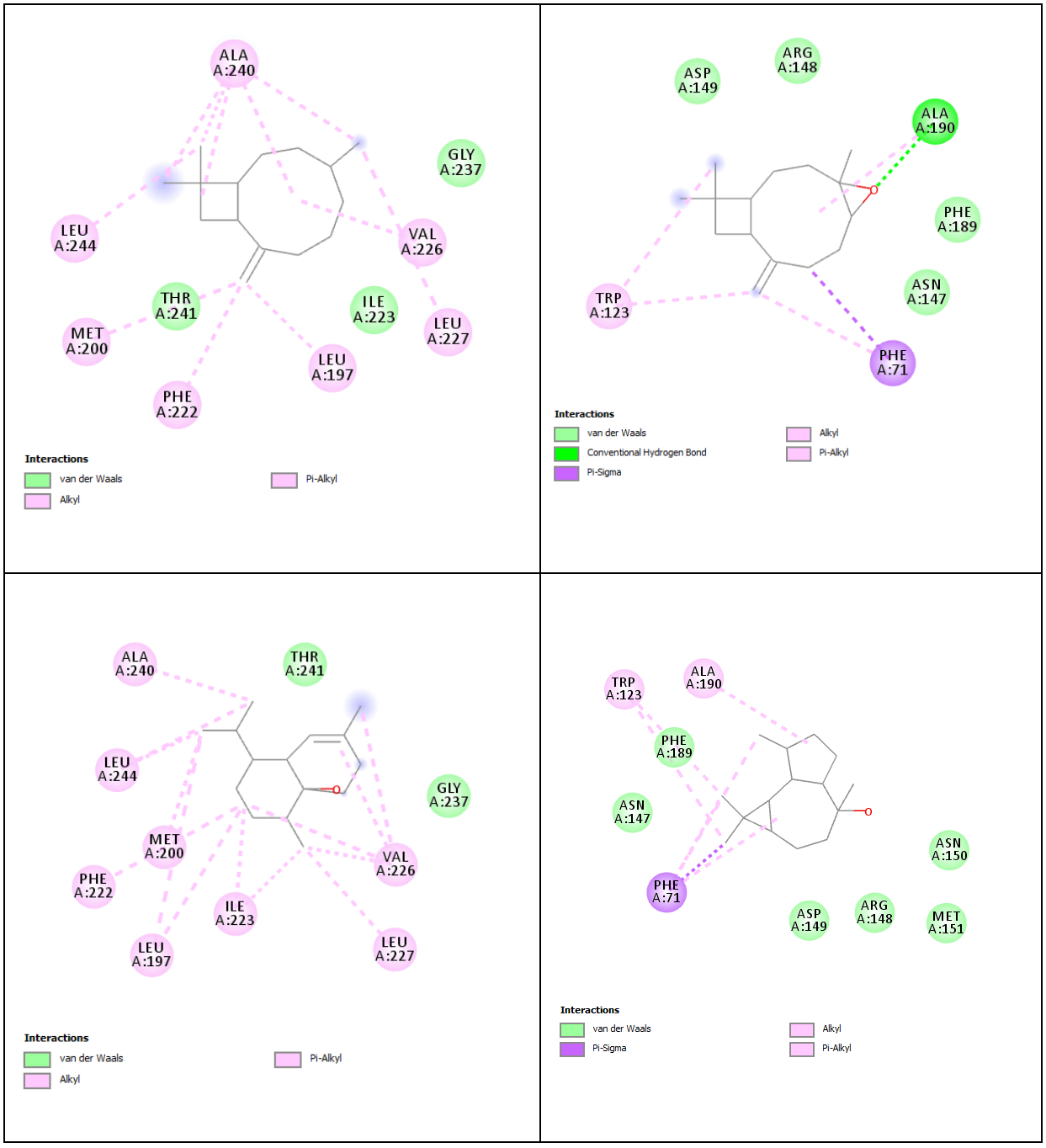

Figure 4b: Molecular docking of Trans (beta)-caryophyllene, caryophyllene oxide, Cubenol and Ledol against the target protein with the PDB id: 3TYE

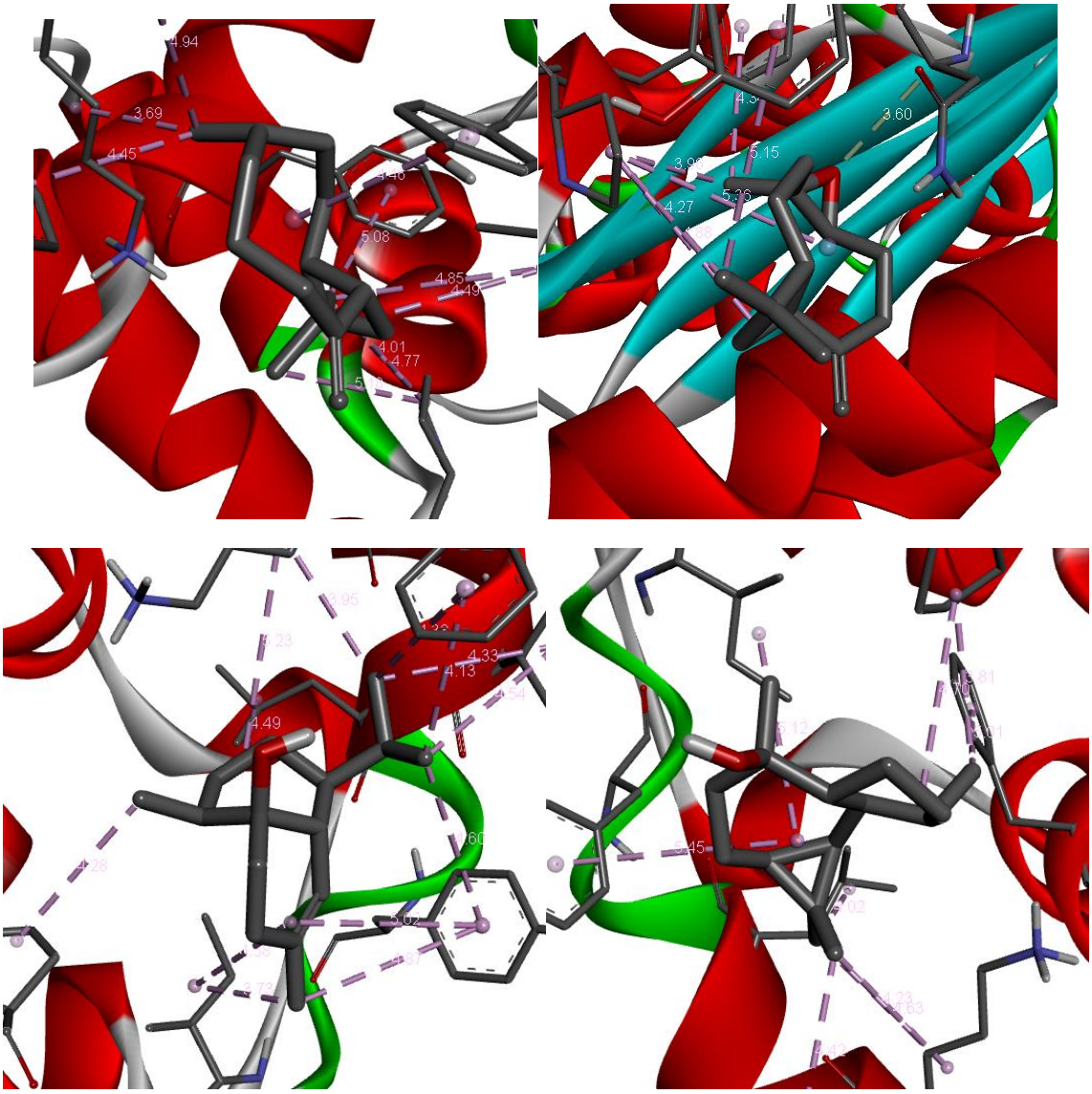

Figure 4c: Molecular docking 3D images of Trans (beta)-caryophyllene, caryophyllene oxide, Cubenol and Ledol against the target protein with the PDB id: 3UDI

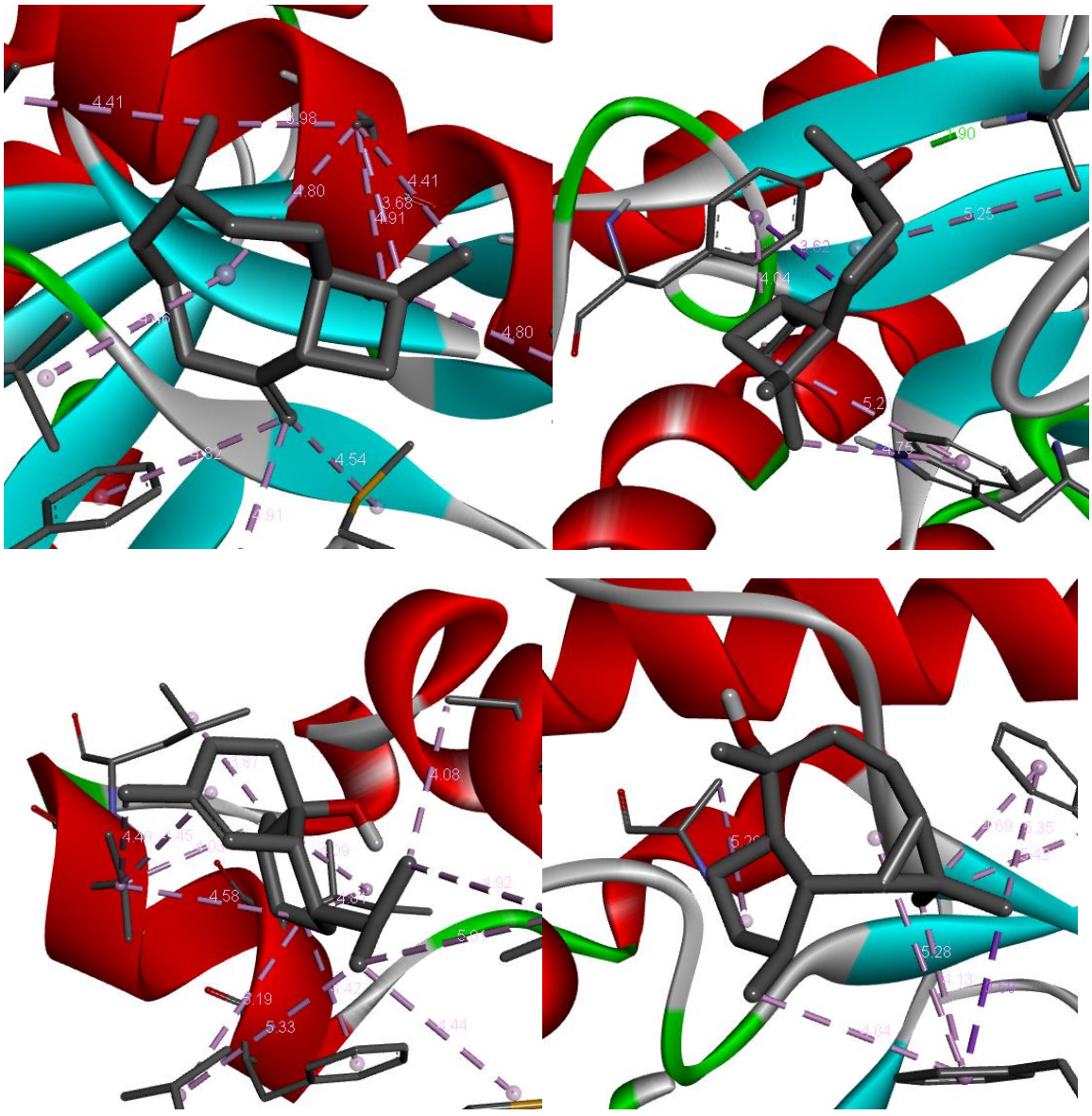

Figure 4d: Molecular docking 3D images of Trans (beta)-caryophyllene, caryophyllene oxide, Cubenol and Ledol against the target protein with the PDB id: 3TYE

Table 2. Antibacterial activity of the essential oil obtained from the flowers of *A. hexapetalus*

Bacterial strain	Zone of Inhibition(mm)		Minimum Inhibitory Concentration(mg)	
	Essential oil	Ampicillin	Essential oil	Ampicillin
Streptococcus pneumonia	16.4	19.5	2.5	2.5
Staphylococcus aureus	15.7	21.5	5.0	2.5
Streptococcus pyogenes	17.5	23.5	2.5	2.5
Pseudomonas aeruginosa	14.5	21.5	5.0	2.5

Table 3. Molecular docking analysis of the essential oil constituents from *A. hexapetalus* against bacterial proteins

Ligands	Docking score 3TYE (Kcal/mole)	Docking score 3UDI (Kcal/mole)
Trans(beta)-caryophyllene	-6.8	-6.9
Carryophyllene oxide	-6.4	-7.0
Cubenol	-6.0	-6.9
Ledol	-6.6	-6.8

4. Conclusion

Using the simple headspace solvent-trapping technique in association with GCMS the components responsible for the odour of the flowers of *A. hexapetalus* flowers were identified. The essential oil obtained from the flowers of *A. hexapetalus by* hydro distillation was analysed by GC-MS, and it lead to the identification of 28 compounds predominantly oxygenated sesquiterpenes (51.91%). Caryophyllene oxide (14.54%), β-caryophyllene (18.69%), cubenol (12.53%) and ledol (11.5%) were the main constituents of the essential oil. The essential oil showed antibacterial activity against bacterial strains *Streptococcus pneumonia*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa.* Molecular docking analysis indicated that the essential oil constituents act as inhibitors of cell well synthesis and nucleic acids synthesis. It can further be explored to use in the fragrances and cosmetics.

Acknowledgements

The authors acknowledge Central Instruments Facility, Karpagam Academy of Higher Education for providing all the equipments, instruments, chemicals and materials during this research work.

Conflicts of Interest

We declare that we have no conflict of interest.

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التركيب الكيميائي، في (سيلكو موليكيولار) دراسات الالتحام الجزيئي والنشاط المضاد للجراثيم في الزيوت الأساسية لزهور *Artabotrys hexapetalus*

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ملخص

عزل المكونات المتطايرة عن زهور ارتابورتيز هيكسابيتاولوس باستخدام عينة تقنية سولفنت–ترابينج والذي تم التعرف عليه من قبل تحاليل جي سي–ام اس. أكبر المكونات هي اثيل اكسيتات 53.6%، ايزوبوتيل اكسيتات (29.4) واثيل بنزوات (14.2). وقد اكتشف أن رائحة المحلول الذي تم الحصول عليه من هذه الطريقة تشبه رائحة الزهور الطازجة. بالإضافة للزيت الأساسي الناتج من أ. هيكسابيتاولوس والذي تم الحصول عليه أول مرة من الهند من قبل جهاز هيدرو ديستيلاشن يوسينج أكليفينجر تايب والذي تم تحليله من قبل جي سي–ام اس. وقد أخرجت النبتة 1.26% من الزيوت الأساسية للزهرة. وقد أسفر التحليل عن التعرف على 28 مكون تشكل 96.17% من الزيت. كما يتكون الزيت العطري في الغالب من اوكسجيناتد سيسكويتربنيس (51.91%) متبوعة ب سيسكويتربنيس (43.31%) وكميات قليلة من مونوتربينس (1.24%) ومكونات آخرى (1.34%). المكونات الرئيسية للزيت الأساسي الذي تم الحصول عليه من الزهور ل أ. هيكسابيتاولوس هي ب–كاريوفيلينى (18.69%)، كاريوفيلليني اوكسيد (14.54%)، كوبنول (12.53%) وليدول (11.5%). وقد أظهر الزيت العطري نشاطًا مضادًا للبكتريا ضد السلالات البكتيرية ستريبتوكوكوز بنومونيا، ستافيلوكوس أوريوس، ستربتوكوكوز بيوجينس أند بسويدوموناس أروجينوسا عارضة منطقة أوف انهيبيشن أوف 16.4، 15.7، 17.5 و14.5 مم وقيمة أم أي سي ب 2.5، 5.0، 2.5، 5.0 مج/مل على التوالي. وقد أشار تحليل موليكولار دوكينج أن مكونات الزيت العطري هي حمض نووي ومثبطات تخليق جدار الخلية. وعليه من المجدي إدخال ذلك في العطور ومستحضرات التجميل.

الكلمات الدالة: *Artabotrys hexapetalus*، الزيوت الأساسية، جي سي–ام اس، سيسكويتربنيس، أس كاريوفيلليني، انتيباكتيريال، الالتحام الجزيئي.

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تاريخ استلام البحث 2020/10/19 وتاريخ قبوله للنشر 2022/1/20.

Jordan Journal of Pharmaceutical Sciences, Volume 15, No. 3, 2022

Impact of Distance Learning on Pharmacy and Pharm.D Undergraduates' during the COVID-19 Pandemic in Jordan

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ABSTRACT

Objective: The study aims to evaluate the impact of distance education on Pharmacy, Pharm.D and postgraduate students' satisfaction and its associated factors during COVID -19 pandemic.

Methods: A cross-sectional web-based survey was distributed online for Pharmacy, Pharm.D and postgraduate Diploma and Master Students across Jordanian universities. Expiratory factor analysis (EFA) and Cronbach's alpha were conducted to examine the validity and the internal consistency of the survey, respectively. .Analysis of Covariance (ANCOVA), Chi square test and t-test were conducted to evaluate the variables associated with students' satisfaction with distance learning.

Results: A total of 860 students completed the survey. The EFA generated a three-factor model including positive impact, negative impact and general impact. The mean scores of the factors were 2.84 (SD=1.03), 2.78 (SD=0.92) and 2.34 (SD=1.22) respectively. Several factors were associated with students' level of satisfaction with distant learning including gender, nationality, university type and field of study.

Conclusion: Distance education had negative impact on Pharmacy and Pharm.D. students' satisfaction, which opens the doors for the necessity to improve the distance education for university students. Variables including gender, nationality, university type and field of study were associated with students' level of satisfaction.

Keywords: The COVID-19, distance learning, satisfaction, impact; pharmacy and pharm. D students, Jordan.

INTRODUCTION

In December 2019, the world started investigating an outbreak of a novel virus named Coronavirus Disease of 2019 (COVID-19), which started from Wuhan, China.(1) The virus spread worldwide and millions of confirmed cases had been recorded and hundreds of thousands of lives had been claimed by the infection(2). The COVID-19 pandemic requires a number of varied restriction strategies as preventative measures to stop or slow down its spread around the world. Therefore, many countries, including Jordan, imposed an overall lockdown that included travel restrictions, quarantines, and schools and universities closure.(3,4) These measures intended are to help in slowing the infection rate and providing time to researchers to come up with effective treatment against COVID-19.(5) This on the other hand obligated activation of distance education and web-based learning for schools and universities. Despite the advantages of e-Learning in

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Received: 16/12/2020 Accepted: 20/1/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.409*

saving the mental and physical health of school and college students, disadvantages have equally been identified, including the lack of interaction between both faculty academics and students, the negative effect on students' communication skills, and the less efficient technique of learning due to remoteness when compared to the face-to-face learning process (6, 7).

An earlier study showed that remote-campus students reported lower academic performance than main-campus ones.(8) On the other hand, other studies reported the opposite and showed that distance education was more effective and successful.(9-11)

Such controversial findings led to many questions about the impact of this time period on learning outcomes and university students' academic performance. Therefore, this study aims to describe students' experiences of distance education and to investigate the impact of distance education on Pharmacy students' satisfaction with the education process during the COVID-19 pandemic. The present study findings should provide insights on and opens doors for improving the web-based teaching environment and hence improving education outcomes.

Aim of study

The aim of this study is to evaluate the impact of distance education on Pharmacy, Pharm. D and postgraduate Diploma and Master students' satisfaction and its associated factors during the COVID -19 pandemic.

Methods

Study design and subjects

A cross-sectional web-based design survey was distributed among first to sixth-year Pharmacy and Pharm.D. in addition to postgraduate students including Diploma and Master students across all universities in Jordan. The study participants were registered in Jordanian universities that are accredited by the Accreditation Council of Higher Education in Jordan. The study received ethical approval (number: (36/132/2020) by the Institutional Review Board Committee at King Abdullah University Hospital / Jordan University of Science and Technology in May 2020.

Study instrument

The survey used in this study was a custom-designed questionnaire that describes participants' demographics including age, gender, nationality, level of study, name of the University, and academic patch. The survey also evaluated students' satisfaction with distance education and its' impact on academic performance and usual daily activities. Ten questions were included in the questionnaire to evaluate the students' satisfaction level with distance learning. The validated questionnaire contained three factors; the first one was "Negative impact", which consists of five questions that discussed the quality of the applied distance education, the fairness of assessment, and the impact of distance education on clinical training. The second factor was the "Positive impact" which included three questions related to students' satisfaction level toward distance learning and whether they enjoyed it or not and the advantage provided by this system in terms of saving transportation time. The final factor was the "general impact" which evaluated how COVID-19 changed the students' study plans and daily activities routine. The score was reversed in questions that evaluated "Negative and General Impact", where a higher mean indicated lower satisfaction with distance education. The questionnaire was developed in English, the official study language at Colleges of Pharmacy in Jordan. The questionnaire was reviewed for face validity by expert academics in the field of pharmacy practice and Pharmacoepidemiology. The Google-formatted survey was piloted on purposely selected twenty Pharmacy and Pharm.D. k students and the results of the pilot study were excluded from the final analysis, and their feedback regarding the clarity and length of the survey was addressed appropriately. The questionnaire included ten 5 points Likert-type scale questions which evaluated the students' satisfaction with distance education. A score of 5 indicated complete satisfaction and the score of one represents complete dissatisfaction, with reverse scoring used for negative questions.

Sample size calculation

The sample size was calculated using the Kish formula for sample size estimation at a 95% significance level and 5% error margin. The estimated sample size was 384. To avoid dropout, 10% of the sample size was added and the target sample size, therefore, was 422. However, a total of 860 undergraduate and postgraduate students were recruited in the present study.

Statistical analysis

Data analysis was conducted using SPSS Version 20. Continuous variables were presented as means and standard deviations (SD), while categorical variables were presented as frequencies and percentages.

Exploratory factor analysis (EFA) was conducted to validate the questionnaire and determine the best model that represents the study data. Kaiser-Meyer-Olkin value (KMO), and Bartlett's Test of Sphericity were conducted to evaluate the suitability of the data for EFA. Communalities were examined, and any item with a commonality that was less than 0.4 was removed from the data. Parallel analysis and scree plot were used to determine the most suitable number of factors for the study data. EPA was conducted using principal-components analysis using varimax rotation; orthogonal rotation was used because the correlations of the produced factors were less than the 0.32 cut-off point. Any item that had a loading below 0.4 in all factors or had a loading of 0.4 or more in multiple factors was excluded. Discriminate validity was evaluated by examining the factor correlation matrix. Cronbach's alpha and Cronbach's alpha if item deleted were calculated to evaluate the internal consistency of each factor.

T-test, chi-square, and analysis of covariance (ANCOVA) were used to evaluate any significant association between participants' demographics and their satisfaction level with distance education.

Results

A total of 860 Pharmacy students completed the questionnaire. As shown in Table 1, the majority of the students were females (67.3%), Jordanians (81.5%), and undergraduate Pharmacy students (60%). No significant difference in the number of the participants in terms of private versus public university was observed in the current study.

Table 1. Demographics of the study participants

		Frequency (Percent)
Gender	Female	108 (37.8)
	Male	178 (62.2)
Degree of Study	BSc.	9 (3.1)
	PhD.	225(78.7)
	MSc.	52 (18.2)
Academic Position	Professor	40 (14)
	Associate Professor Assistant Professor	83 (29)
	Teaching Assistant	103 (36)
	Teacher	10 (3.5)
		50 (17.5)
Employment Status	Part Time	12 (4.2)
	Full Time	274 (95.8)
Specialty	Medical	122 (42.7)
	Social Sciences	107 (37.4)
	Engineering, IT, Science	57 (19.9)
Field of Education	Scientific	226 (79)
	Non-Scientific	60 (21)

		Frequency (Percent)
Have you operated online teaching before the COVID-19 pandemic?	No Yes	168 (58.7) 118 (41.3)
Have you received training for online teaching?	No Yes	143 (50) 143 (50)
Have you attended any courses as a trainee through the internet?	No Yes	123 (43) 163 (57)
Age		44.30 (9.652)
Number of years of online teaching		3.49 (3.302)
Number of years of teaching experience		11.31 (8.037)

The KMO test result was 0.82 and Bartlett's Test of Sphericity was $\chi 2\ (45) = 3246.57$, $p < 0.01$ indicating that the study data are suitable for factor analysis. Scree plots (figure 1) and parallel analysis indicated that the three-factor model was the most suitable representation of the study data; the three factors are "Positive impact", "Negative impact" and "General impact". As Table 2 shows, factor loadings for all the ten items were higher than 0.04 and the item "I'm aware that online classes are the only way to continue the semester, but need better strategies", in the "Negative impact" factor had the lowest loading (0.69) and the lowest communality (0.57). All three factors had a high Cronbach's alpha (above 0.8) indicating acceptable internal consistency; also, when applicable, deleting an item will not improve the reliability of the factor. The mean of the three factors "General impact", "Positive impact" and "Negative impact" was 2.34 (SD=1.22), 2.84, (SD=1.03) and 2.78, (SD=0.92) respectively, and the total mean of the three factors was 2.66 (SD=0.64). The item "the online education is the worst thing happened" had the higher mean =3.10, SD=1.20), whereas the item "the effect of COVID-19 on your daily routine" had the lowest mean 2.32, SD=1.35.

Table 2. The mean and standard deviation for each questionnaire item and for the total items

	Mean (Std)
1.The level of my interactions with students in the online course is higher than in a traditional face-to-face class.	2.25 (1.09)
2.The flexibility provided by the online environment is important to me.	3.44 (1.10)
3.My online students are actively involved in their learning.	2.66 (1.10)
4.I incorporate fewer resources when teaching an online course as compared to traditional teaching.	3.47 (1.17)
5.The technology I use for online teaching is reliable.	3.65 (0.94)
6.I have a higher workload when teaching an online course as compared to the traditional one.	1.81 (0.96)
7.I miss face-to-face contact with students when teaching online.	1.76 (0.90)
8.I do not have any problems controlling my students in the online environment.	2.95 (1.22)
9.I look forward to teaching my next online course.	2.66 (1.09)

	Mean (Std)
10. My students are very active in communicating with me regarding online course matters.	2.98 (1.11)
11. I appreciate that I can access my online course any time at my convenience.	3.48 (0.98)
12. My online students are more enthusiastic about their learning than their traditional counterparts.	2.14 (0.93)
13. I have to be more creative in terms of the resources used for the online course.	2.17 (0.91)
14. Online teaching is often frustrating because of technical problems.	2.48 (0.97)
15. It takes me longer to prepare for an online course on a weekly basis than for a face-to face course.	1.88 (0.91)
16. I am satisfied with the use of communication tools in the online environment (e.g., chat rooms, threaded discussions, etc.).	3.4 (0.94)
17. I am able to provide better feedback to my online students on their performance in the course.	2.71 (1.05)
18. I am more satisfied with teaching online as compared to other delivery methods.	2.38 (1.08)
19. My online students are somewhat passive when it comes to contacting the instructor regarding course related matters.	2.5 (0.96)
20. It is valuable to me that my students can access my online course from any place in the world.	3.96 (0.73)
21. The participation level of my students in the class discussions in the online setting is lower than in the traditional one.	2.15 (0.97)
22. My students use a wider range of resources in the online setting than in the traditional one.	2.81 (1.08)
23. Technical problems do not discourage me from teaching online.	3.16 (1.14)
24. I receive fair compensation for online teaching.	3.33 (1.08)
25. Not meeting my online students face-to-face prevents me from knowing them as well as my on-site students.	3.56 (1.57)
26. I am concerned about receiving lower course evaluations in the online course as compared to the traditional one.	2.6 (0.98)
27. Online teaching is gratifying because it provides me with an opportunity to reach students who otherwise would not be able to take courses.	2.88 (1.03)
28. It is more difficult for me to motivate my students in the online environment than in the traditional setting.	1.97 (0.89)
Total Items	2.76 (0.5)

As shown in Table 3, there was a significant association between different variables and the mean of each factor and the overall factors. For example, the total impact (the mean of the three factors) was significantly associated with the field of study, nationality, gender, and college year. These results were also confirmed when ANCOVA was conducted, as the result of the analysis indicated that several factors were significantly associated with total impact including gender, nationality, university type, and field of study.

Table 3. Questionnaire Subgroup Satisfaction Scores

		Mean (SD)
Gender	Female	2.75 (0.50)
	Male	2.76 (0.50)
Degree of Study	BSc.	2.61 (0.38)
	PhD.	2.76 (0.51)
	MSc.	2.78 (0.50)
Academic Position	Professor	2.85 (0.43)
	Associate Professor	2.78 (0.48)
	Assistant Professor	2.70 (0.56)
	Teaching Assistant	2.71 (0.34)
	Teacher	2.77 (0.47)
Employment Status	Part Time	1.5 (0.37)
	Full Time	1.9 (0.38)
Specialty	Medical	1.49 (0.08)
	Social Sciences	1.97 (0.12)
	Engineering, IT, Science	2.47 (0.11)
Field of Education	Scientific	2.77 (0.50)
	Non-Scientific	2.70 (0.48)
Have you operated online teaching before the COVID-19 pandemic?	No	2.71 (0.49)
	Yes	2.82 (0.51)
Have you received training for online teaching? *	No	2.69 (0.49)
	Yes	2.82 (0.50)
Have you attended any courses as a trainee through the internet? *	No	2.62 (0.50)
	Yes	2.86 (0.47)

***** P value <0.05

Discussion

The present study enlightens the impact of distance education on Pharmacy, Pharm. D and postgraduate Diploma and Master Students' satisfaction during COVID-19 pandemic. This study formulated and validated a questionnaire to evaluate students' level of satisfaction with distance education and analyzed the factors that may influence their satisfaction. Students' satisfaction towards distance learning methods may be influenced by several factors such as internet server capacity, internet connection speed, and examination security.(12) Such different variables might explain the contradicting findings with of students' level of satisfaction with distance learning.(13-15) As shown in the results, the majority of participated students reported low satisfaction with the web-based learning, even though the students had the same content, the same form of evaluation, and the same level of information delivery in both distances and on-campus education. Many students in the present study described distance education as the worst thing that happened (mean of that question=3.10, SD=1.20), and many considered that the education quality during online education decreased (mean=2.73, SD= 1.18). The current study finding is consistent with previous research findings, which reported that Pharmacy students in the remote campus were less satisfied with the education process and

had significantly lower course scores when compared with students who had campus-based learning.(8) A meta-analysis study reported that high-education students were more satisfied with live campus-based courses when compared to distance education formats. This may be attributed to the insufficient quality of services provided by e-learning which require computer access, (16) in addition to the technical and internet access problems or the low students' technical skills.(17) Moreover, students' satisfaction level may be influenced by the lack of interactivity nature of the online learning material which is limited to videos and written e-mails.(18) On the other hand, many studies demonstrated more students' satisfaction with the e-learning process,(19-20) where they could check emails and the University website and were capable to have access to the Moodle platform at home to complete their assignments on daily basis. Furthermore, the e-learning process enhances students' active participation by verifying and mentoring students' access to completed the required assignments.(11)

The results of this study indicated that students were not satisfied with the clinical training received during the pandemic, and they thought that they were deprived of getting field-related knowledge and the information they need. The lack of direct interaction with other healthcare professionals, which could negatively impact the inter-professional learning experience for the clinical training of the students,(21) could justify this finding.

Consistent with a previous research finding,(22) advanced-stage students with prior and frequent online experiences during their academic educational journey such as master degree students, showed significantly higher satisfaction level and were more able to learn from different video styles, when compared with other earlier stage students. Results also showed that males were more satisfied with two of the three factors than females, but the reasons are not clear yet.

On the "General impact" questions, the students confirmed that the COVID-19 and distance learning made significant changes in their daily routine and study plans. However, this finding was not surprising because this is the first time they applied distance learning in full-term with no prior preparation strategies.

The current study findings shed the light on improving the e-learning technique that provides distinctive academic services and ensuring that each component of the integrated educational system contributes to improving the student learning environment.(23) One of the strongest recommendations to improve distance learning is to create clear and unobstructed visual materials while the instructor is also simultaneously visible.(22)

Limitations

The sample is web-based thus there is a subject selection bias and generalizability is not warranted. Other factors such as student GPA, geographical location, number of family member vs. number of devices, grade obtained and others which could potentially impact student satisfaction were not evaluated in the present study. Finally, the measure of satisfaction might have improved by now or get more accepted, thus the study findings might not resilient over time.

Conclusion

The students' low satisfaction with distance education reported in the present study opens doors for distance education improvement and supports more learner-focused approaches to improve the web-based teaching environment and hence improving education outcomes. It is expected that the outcomes of this study will help decision-makers understand the challenges that affect students' motivation toward online learning in order to implement future interventions which match students' expectation in a step to improve the online learning outcomes.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contribution

AJ, WA, TL and AA conceived and designed the study.

AJ, TL, DA and AA conducted research, provided research materials, and collected and organized data. AJ, WA and DA analyzed and interpreted the data. TL, AA and DA wrote the initial and final drafts of the article and provided logistic support. AJ and WA performed the critical revisions in the manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Acknowledgement

The authors wish to thank all Pharmacy and Pharm. D students in Jordan for their prompt response in filling the present study survey

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تأثير التعلم عن بعد على طلاب الصيدلة والصيدلة السريرية الجامعيين خلال جائحة كوفيد -19 في الأردن

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ملخص

الهدف: تهدف الدراسة الحالية إلى تقييم تأثير التعليم عن بعد على رضا طلاب الصيدلة والصيدلة السريرية والعوامل المرتبطة به خلال جائحة كوفيد-19.

الطُرق: تم توزيع مسح مقطعي على شبكة الإنترنت على طلاب الصيدلة والصيدلة السريرية في مختلف الجامعات الأردنية. تم إجراء تحليل عامل الاستكشاف وCronbach's alpha لفحص الصلاحية والاتساق الداخلي للمسح على التوالي. تم إجراء تحليل التباين المشترك (ANCOVA) واختبار مربع كاي واختبار تي لتقييم المتغيرات المرتبطة برضا الطلاب عن التعلم عن بعد .

النتائج: أكمل الاستبيان 860 طالبًا. أنتج تحليل عامل الاستكشاف نموذجا من ثلاثة عوامل يتضمن التأثير الإيجابي والأثر السلبي والأثر العام. كان متوسط درجات العوامل 2.84 (1.03)، 2.78 (0.92) و2.34 (1.22) على التوالي. ارتبطت عدة عوامل بمستوى رضا الطلاب عن التعلم عن بعد بما في ذلك الجنس والجنسية ونوع الجامعة ومجال الدراسة.

الخلاصة: كان للتعليم عن بعد تأثير سلبي على رضا طلاب الصيدلة والصيدلة السريرية، مما يفتح الأبواب لضرورة تحسين التعليم عن بعد لطلاب الجامعة. ارتبطت المتغيرات بما في ذلك الجنس والجنسية ونوع الجامعة ومجال الدراسة بمستوى رضا الطلاب.

الكلمات الدالة: كوفيد-19، الدراسة عن بعد، رضا، تأثير، طلاب الصيدلة والصيدلة السريرية، الأردن.

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تاريخ استلام البحث 2020/12/16 وتاريخ قبوله للنشر 2022/1/20.

Public Perception of Pharmacist's Role during COVID-19 Outbreak in Jordan

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ABSTRACT

Background: The pandemic COVID-19 requires collaborative teamwork by all healthcare professionals including Pharmacists who could help in combating epidemic diseases by providing several pharmaceutical services. Nevertheless, public perception of pharmacist's role in providing health service is controversial

Methods: A cross-sectional web-based design validated survey of 25 items was used to explore patients' opinion about pharmacist's ability to provide different health services during COVID-19 pandemic. Exploratory factor analysis (EFA) was conducted to evaluate the best model for the questionnaire. The association between different demographic variables and awareness about pharmacist's role was evaluated using Pearson correlation, Mann-Whitney u test and Kruskal–Wallis one-way analysis of variance.

Results: A total of 668 persons participated in the study. The mean (SD) of the respondent questionnaire scores was 97.1 (12.6) and the possible maximum score was 115 (12.9). Higher awareness score was associated with increased age, female gender, lower educational level, living out of Amman the capital, being college or university student or being employed in medical field.

Conclusion: The positive public perception toward pharmacist role shown in the present study enlighten the need to expand pharmacist role to be more engaged in providing different health services during the disaster or normal conditions.

Keywords: COVID-19, Public, Perception, Pharmacist, Awareness, Health service, Jordan.

INTRODUCTION

Coronavirus disease (COVID-19) is a new breakthrough viral respiratory disease that is characterized by symptoms similar to that of the common cold including fever, fatigue, dry cough and shortness of breath[1]. However, the degree of these symptoms varies from one patient to another, ranging from mild to severe acute respiratory distress syndrome, and in some cases it can cause death[2,3] The origin of COVID-19 comes from bats that have mutated with this virus and was then transmitted to the humans [4]. This virus is very contagious and can be transmitted between people in an uncontrollable way through respiratory droplets and secretion, and by contaminated inanimate surfaces of plastic, glass or metal. The disease could be symptomatic or asymptomatic[5,6] It affects children, adults and elderly people but it is more prominent in the elderly people, especially those with chronic diseases such as diabetes and hypertension [7].

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Received: 7/6/2021 Accepted: 20/1/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.410*

In February 2020, the World Health Organization (WHO) termed this disease COVID-19, which is discovered in 2019 and the first case was identified in China.[2,3,8]. On March 11th 2020, COVID-19 was announced a global pandemic disease and most countries around the world have registered COVID-19 cases within short period of time [9]. The epidemic COVID-19 requires collaborative teamwork by all healthcare professionals including pharmacists, who can play a fundamental and unique role to improve patient care, especially during this COVID-19 pandemic[10,11].

On March 19, 2020, the International Federation of Pharmacy (FIP) guideline reported several recommendations for dealing with a COVID-19 outbreak, including interventions and patient counseling that pharmacists should provide during this serious situation [12]. Pharmacists could help in epidemic diseases such as COVID-19 by providing a lot of services such as guaranteeing the availability and timely provision of the safest and most effective therapy, in which they must plan for, identify, and mitigate drug shortages, especially during this pandemic[13]. Pharmacists could educate patients and the public community about the effective strategies required to prevent further spread of the infection such as optimal hand hygiene, social distancing, staying home if having respiratory symptoms and symptoms relief [13]. Nevertheless, public perception of pharmacist's role in providing health service in pandemic or other health problem is controversial. While some studies reported public awareness of the pharmacist role [14,15,16], other studies failed to report similar finding [17,18,19].

Aim of the study

The aim of this study therefore was to explore public perception of pharmacist role during COVID 19 outbreak. The present study is the first one in Jordan to evaluate public perception of pharmacist role during the COVID 19 outbreak. Findings of the present study will help to investigate how do the community members understand and perceive the pharmacists' role during COVID 19 outbreak, providing a guide on how to reinforce and improve this role during any future crisis.

Methods

Study design and participants

A cross-sectional web-based design survey was distributed anonymously online using social media, with the Facebook as platform of the study on 12 April 2020. Adults older than 18 years and living in Jordan were asked to participate in the study with emphasis on the right to withdraw at any time. The initial questionnaire was developed in English and translated into Arabic, the official language in Jordan, using forward-backwards translation, then face validity was evaluated. The content validity of the questionnaire was evaluated by a panel of experts in clinical pharmacy practice and pharmacoepidemiology. In order to improve the reliability and clarity of the survey, the questionnaire was formatted into Google form and piloted among twenty purposely selected society members who were excluded from the main study.

Study instrument

In addition to the socio-demographics, the questionnaire was composed of 25 items describing pharmacist role in implementing different services during the COVID-19 pandemic such as providing sufficient information about causes, symptoms, prevention and management of COVID-19, being able to discover and develop the necessary treatment for COVID-19, being reliable and accessible resource for providing pharmaceutical advice about the most appropriate therapeutic regimen during a public health emergency such as the pandemic covid-19, reviewing and interpreting information and studies related to the Corona epidemic for other healthcare team members and being responsible for ensuring the availability of safest and most effective treatment [13]. The study participants were asked about their opinion on pharmacist's ability to perform each service on a 5-likert scale ranging from strongly disagree to strongly agree.

Ethical approval

The study received ethical approval from the Scientific

Research and Ethics Committee at Jordan University of Science and Technology.

Statistical analysis

The SPSS statistical package version 25 was used to analyze the data. Continuous data was expressed as mean and SD, while categorical variables were expressed as frequencies and percentages. Kaiser-Meyer-Olkin value (KMO), and Bartlett's Test of Sphericity were performed to evaluate the suitability of the data for factor analysis. Exploratory factor analysis (EFA) was conducted using principal-components analysis to evaluate the most suitable model for the study data. Scree plot was produced to evaluate the suitable number of factors to be included in the model. Communalities were produced and only items above 0.35 were retained. Cronbach's alpha for each factor, Corrected item-total correlation, and Cronbach's alpha if item deleted were calculated to evaluate the internal consistency of the questionnaire. The ceiling and flooring effect was evaluated by calculating the percentage of participant who had the maximal or minimal possible scores, the accepted range to exclude these effect is below 15%[20]. Pearson correlation, Mann-Whitney u test and Kruskal–Wallis one-way analysis of variance were used to examine the correlation between different confounding factors and the respondents' scores in the questionnaire.

Results

As shown in Table 1, a total of 668 participants completed the study questionnaire. The mean age of the sample was 30.18 (10.679) and most of them were female (67.8%), had education level of bachelor's degree (74.4%), lived outside Amman (73.3%), were insured (79.2%), had income below 800 JD (64.2%) and were college or university students (32.5%). The participants' mean number of children was 1.33 (±2.51). Social media was the most recognized method (37.9%) to spread awareness about COVID-19 by pharmacist KMO and Bartlett's Test of Sphericity were 0.956 & $\chi 2$ (406) = 21975.94, $P < 0.01$, indicating the suitability of data for expiratory factor analysis (EFA). Scree plots indicated that a one factor model is the most suitable model for the study data (Figure 1).

Table1: Demographic characteristics of the study participants (n=668)

Variables	Number of participants (%)
Age (mean, SD)	30.18 (10.68)
Number of Children (mean, SD)	1.33 (2.51)
Gender	
Male	215 (32.2%)
Female	435 (67.8%)
Education Level	
High school	33 (4.9%)
Bachelor's degree	497 (74.4%)
Higher education (Master or PhD)	138 (20.7%)
Social Status	
Married	302 (45.2%)
Not married	366 (54.8%)
Current Job	
Unemployed	131 (19.6%)
Medical sector (doctor, nurse, dentist, pharmacist, medical laboratory)	119 (17.8%)
Outside the medical sector (government employee, private sector, self-employment)	182 (27.2%)
College or university student	217 (32.5%)
Retired	19 (2.8%)

Variables		Number of participants (%)
Income level		
	Less than 800 dinars per month	429 (64.2%)
	800-1600 dinars per month	161 (24.1%)
	1601-2400 dinars per month	47 (7%)
	More than 2,400 dinars per month	31 (4.6%)
Place of Living		
	Inside Amman	176 (26.3%)
	Outside Amman	492 (73.7%)
Health Insurance		
	Yes	529 (79.2%)
	No	139 (20.8%)
Best way for a pharmacist to spread awareness		
	Social media	253 (37.9%)
	Make educational leaflet	24 (3.6%)
	Explanatory videos	106 (15.9%)
	TV screen channels	31 (4.6 %)
	Directly to customers in the pharmacy	254 (38%)

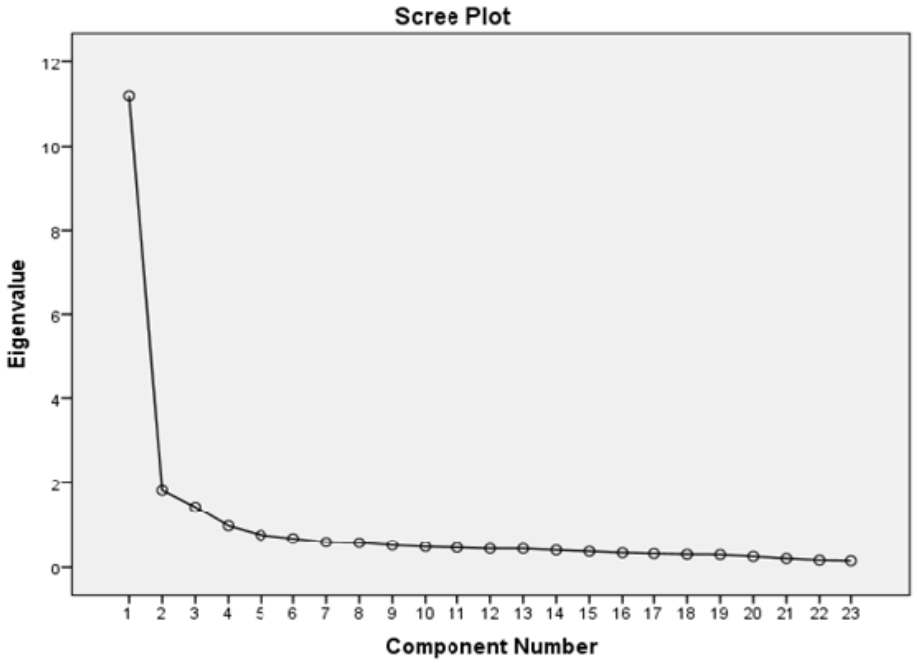

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Figure 1: Scree plot for factor analysis

Items 24 (The home delivery service of the drug was very useful during the Corona crisis), and 25 (Home visits by pharmacists are needed to give advice about treatments) were removed due to low communalities (>0.35). EFA was re-run after excluding items 24 and 25. The best model for the study data was a one factor model composed of 23 items as shown in Table 2.

Table 2: Questionnaire scores for each group and the significance

Variables		Total Score (mean, SD)	*P* value *
Age (mean, SD)			< 0.01
Number of Children (mean, SD)			N/S
Gender			0.01
	Male	94.58 (15.31)	
	Female	98.30 (10.91)	
Education Level			< 0.01
	High school	98.42 (9.58)	
	Bachelor's degree	98.42 (11.29)	
	Higher education (Master or PhD)	92.04 (16.06)	
Social Status			< 0.01
	Married	94.93 (12.85)	
	Not married	98.89 (12.13)	
Current Job			< 0.01
	Unemployed	94.87 (10.27)	
	Medical sector (doctor, nurse, dentist, pharmacist, medical laboratory)	99.18 (11.55) 92.63 (15.12)	
	Outside the medical sector (government employee, private sector, self-employment)	101.04 (10.57) 97.37 (12.61)	
	College or university student		
	Retired		
Income level:			N/S
	Less than 800 dinars per month	98.00 (11.02)	
	800-1600 dinars per month	95.69 (14.94)	
	1601-2400 dinars per month	96.89 (13.67)	
	More than 2,400 dinars per month	92.35 (16.65)	
Place of Living			< 0.01
	Inside Amman	94.06 (15.53)	
	Outside Amman	98.19 (11.20)	
Health Insurance			N/S
	Yes	96.74 (12.62)	
	No	98.49 (12.49)	
Best way for a pharmacist to spread awareness			N/S
	Social media	93.21 (13.84)	
	Make educational leaflet	98.37 (10.77)	
	Explanatory videos	98.39 (16.37)	
	TV screen channels	96.48 (13.78)	
	Directly to customers in the pharmacy	97.42 (11.37)	

**P* value measure association between total awareness score and sociodemographic characteristics.

Item 16 had the lowest communality (0.38) and the lowest loading (0.62). While the highest communality and factor loadings were for item7 (0.61 and 0.78 respectively). The highest two means were item 17 (4.60) and item (4.41), while item 3 (3.97) and item 5 (4.00) had the lowest means. Also, item 3 had the highest variability

between participants (SD=1.00), while item 23 had the lowest variation (SD = 0.66). The factor's Cronbach's alpha was 0.95 and deleting any items would not increase the internal consistency of the model. The percentages of the participants who scored the highest or lowest possible scores were less than the threshold point of 15%.

The mean (SD) of the total respondent questionnaire scores was 97.1 (12.6) and the highest score was 115 (12.9). Results of Pearson correlation, Mann-Whitney U test or Kruskal–Wallis one-way analysis of variance showed that age, gender, education level, social status, current job, and place of living were significantly associated with the questionnaire scores (Table 3). For example, females had significantly higher scores when compared with males (P=0,01). Higher education group had significantly less score when compared with other education groups (P<0.01), higher scores were also reported by university students and participants living in Amman (P< 0.01).

Table 3: Item loadings, corrected total correlation and Cronbach's Alpha If Item Deleted

Questions	Factor Loadings	Corrected Item-Total Correlation	Cronbach's Alpha If Item Deleted	communalities	mean (SD)
Q1: Pharmacists have a key role in limiting the spread of the Corona epidemic.	0.67	0.64	0.95	0.45	4.12 (0.87)
Q2: The pharmacist has an important role in alleviating the negative consequences caused by Corona's disease.	0.73	0.71	0.95	0.53	4.10 (0.81)
Q3: The pharmacist's role in addressing the epidemic and corona treatment and to mitigate the negative consequences no less important than the role of members of the medical team, such as doctor and nurse	0.65	0.62	0.95	0.42	3.97 (1.00)
Q4: The pharmacist has sufficient capacity to provide complete and sufficient information about the Corona epidemic.	0.71	0.68	0.95	0.51	4.10 (0.88)
Q5: Pharmacists have a primary role in reviewing and interpreting information and studies related to the Corona epidemic of fellow doctors and nurses.	0.69	0.66	0.95	0.48	4.00 (0.85)
Q6: Pharmacists are a reliable resource that all people can access during a public health emergency, such as the Corona epidemic	0.72	0.69	0.95	0.52	4.01 (0.91)
Q7: The pharmacist has an important role in educating their patients and the public about effective efficacy to prevent infection acquisition and spread (such as ideal hand hygiene and avoiding contact and staying at home if respiratory symptoms appear).	0.78	0.75	0.95	0.61	4.36 (0.74)
Q8: The pharmacist has an important role in providing people (whether or not he is infected) with drug information related to drugs used to treat the Corona epidemic, and the side effects of their use.	0.76	0.72	0.95	0.57	4.32 (0.76)

Questions	Factor Loadings	Corrected Item-Total Correlation	Cronbach's Alpha If Item Deleted	communalities	mean (SD)
Q9: Pharmacists have an important role in ensuring that the safest and most effective treatment is available and available in a timely manner, in addition to ensuring access to these treatments and other medicines that may be deficient due to the epidemic.	0.77	0.74	0.95	0.59	4.24 (0.78)
Q10: Pharmacists must act proactively and determine the effectiveness of treatment alternatives.	0.65	0.61	0.95	0.42	4.16 (0.80)
Q11: The pharmacist has a primary role in discovering and developing the necessary treatment for the Corona epidemic.	0.66	0.63	0.95	0.44	4.04 (0.92)
Q12: The pharmacist has an important role in providing the following information about the Corona epidemic: What is an epidemic?	0.68	0.65	0.95	0.47	4.19 (0.79)
Q13: The pharmacist has an important role in providing the following information about the Corona epidemic: Methods of transmission of the epidemic	0.68	0.64	0.95	0.46	4.34 (0.74)
Q14: The pharmacist has an important role in providing the following information about the Corona epidemic: Symptoms of the epidemic	0.67	0.63	0.95	0.45	4.34 (0.75)
Q15: The pharmacist has an important role in providing the following information about the Corona epidemic: Epidemic prevention methods	0.68	0.64	0.95	0.46	4.41 (0.71)
Q16: The pharmacist has an important role in providing pharmaceutical services remotely to prevent the spread of the Corona epidemic, (such as delivering the medications that the patient needs to the home, helping people to take the appropriate medications and determining the doses needed for the medications by communicating with the pharmacist by phone or social networking sites).	0.62	0.57	0.95	0.38	4.37 (0.71)
Q17: The pharmacist has an important role in providing the following information about the Corona epidemic: Causes of Corona epidemic	0.65	0.61	0.95	0.42	4.60 (0.93)
Q18: The pharmacist is able to provide the correct and necessary pharmaceutical advice and indicate reliable sources to obtain the correct information related to the Corona epidemic.	0.75	0.72	0.95	0.57	4.30 (0.73)

Questions	Factor Loadings	Corrected Item-Total Correlation	Cronbach's Alpha If Item Deleted	communalities	mean (SD)
Q19: The pharmacist has an important role in helping doctors and nurses choose the appropriate pharmaceutical form of the medications so that they help limit unnecessary entry into the patient's room.	0.72	0.68	0.95	0.52	4.25 (0.77)
Q20: A pharmacist is an expert in drug information and evaluation of studies related to new treatments or the use of drugs available in the treatment of the Corona epidemic and determining the correct doses for these drugs.	0.74	0.71	0.95	0.55	4.23 (0.80)
Q21: One of the main tasks of pharmacists is to plan the deficiency of drugs, identify them and reduce them during the spread of the epidemic, because the shortage of medicines can lead to prescribing suboptimal treatment and therefore may cause harm to the patient.	0.66	0.62	0.95	0.44	4.30 (0.71)
Q22: Pharmacists working in community pharmacies can help in detecting cases infected with the epidemic by reporting cases with symptoms similar to the symptoms of the epidemic, or telling the patient that an examination is necessary to make sure there is no infection.	0.68	0.65	0.95	0.47	4.26 (0.75)
Q23: The pharmacist has a major role in providing people with their medical needs during the Corona crisis	0.69	0.65	0.95	0.48	4.31 (0.66)

Discussion

Coronavirus disease (COVID-19) is a new widely contagious disease [5,6], that claimed the lives of hundreds of thousands around the world [9], and lead to great economic losses [21]. This great impact of COVID-19 on the global health and economy requires a collaborative teamwork by all healthcare professionals including pharmacists who could help in combating epidemic diseases by providing several pharmaceutical services [10]. Nevertheless, several studies indicated a lack of awareness of the role of pharmacists in public health activities [19,22]. Therefore, there is a need to assess the perception of the public on the role pharmacists may play during a pandemic such as COVID-19. The present study is the first one in Jordan to evaluate public perception of pharmacists' role during the COVID-19 outbreak and demonstrated the importance of this role.

This study validated a tool to measure the public awareness regarding the role of pharmacists during a pandemic such as COVID-19, the results of the factor analysis indicated that all the questions included in the final version after excluding items 24 (The home delivery service of the drug was very useful during the Corona crisis), and 25 (Home visits by pharmacists are needed to give advice about treatments) were highly correlated and loaded in single factor, in addition the Cronbach's alpha result indicated high internal consistency and reliability of the questionnaire.

Although the responses to items that evaluated the perception of the public regarding the general role of pharmacist were consistent, when asking about specific services including home delivery and house visits the responses of the participants varied which may be related to different variables including respondent location

As reported previously, the practice of pharmacy has expanded from merely medicine despising to providing a wide variety of health-related services [23,24,25]. Community pharmacists are the most accessible healthcare professionals [26,27] and have an essential role in connecting physicians with patients [28,29]. The curfew declared in Jordan to limit the spread of COVID-19 limited the ability of the public to reach a pharmacy, which raised a need to provide pharmaceutical services remotely including delivering necessary medicines, particularly for chronic conditions, and provide patient consulting services through a variety of techniques including phone calls, mobile applications and the internet to reduce patients' unnecessary visits to the pharmacy. The remote pharmaceutical services have also been applied in many countries including China [23], which launched the "Online Pharmaceutical Monitoring" service, which is an online pharmaceutical service model that uses WeChat Application with smart mobile during COVID-19 pandemic [30]. In agreement with a previous study findings[23,30], the results of this study indicated that the general public understands the importance of the remote services provided by pharmacist during the COVID-19 pandemic and looks favorably in the important role pharmacist play in providing necessary medication and other necessary COVID-19 preventive products including masks and sanitizers, in addition to providing reliable patient counseling services.

Furthermore, the results of this study show that the general public perceived pharmacists as a reliable source of information about COVID-19 identity, disease symptoms, methods of transmission and management of the disease. The present study also showed that the general public believed in pharmacists' role in improving patients and public awareness about the effective methods to prevent the spread of COVID-19 such as ideal hand hygiene, mask handling and social distancing [30]; which could help in reducing the spread of COVID-19 [31].

The results of this study showed that the general public appraised the necessary role of pharmacists in the interpretation of the latest COVID-19 related studies and in the management of COVID-19. This role was reported in previous studies that highlighted the important pharmacist role in evaluating the different treatment strategies[30,32]. The pharmacist could also play an active role in reducing vaccine hesitancy reported in previous studies[33,34]and correct different misconception about COVID-19[35]

The present study shows that females had significantly higher perception scores about pharmacists' when compared with males (P=0.01). Awad et al. reported that females chose the pharmacist as the first person to contact in minor disease significantly more than males (P= 0.008) [17]. Awad et al. also found that participants aged ≥ 40 years had a more positive view regarding the pharmacists' role in public health when compared with younger participants[17], which is in agreement with the present study findings.

Earlier research studies reported significant association between education level and the perception toward different pharmaceutical services provided by the pharmacists [36,37] The majority of the present study participants had at least a bachelor's degree (95.1%), which could justify the positive views found towards the pharmacist's role in this study.

In agreement with earlier studies which showed that customers from rural areas had a longer conversations with the pharmacists and discussed wider range of health-related topics [38,39,40,41], people who live outside Amman tended to appreciate the pharmacist's role more than those who live inside Amman. This may be attributed to the socio- economic status of the customers, the volume of

business the pharmacy has and having more independent pharmacies rather than chain pharmacies [42].

Study limitations

The main limitation of this study was that only those who were literate and had a connection to the internet were able to participate in the study. Also, a bias may result from the possibility that the participants in this study were more aware or interested in pharmacists' role than those who did show interest to complete the questionnaire. Lastly, the study respondents were mainly young people. However, the Jordanian society is characterized by a youthful status with only 4% are 65 years and above.

Conclusion

In conclusion, this study validated a tool to evaluate Jordan public's perception on the role and satisfaction on the services provided by the pharmacists during COVID-19 epidemic. Public had a positive perception regarding the roles and responsibilities of the pharmacists during COVID-19 epidemic, and were satisfied with different pharmaceutical services provided by the pharmacists including remote patients' education and spreading awareness about COVID-19. The current study demonstrates how do the community members understand and perceive the pharmacists' role during COVID 19 outbreak, providing a guide on how to reinforce and improve this role during any future crisis

Acknowledgements

The authors wish to thank the research ethics committee and the institutional review board at Jordan University of science and Technology for giving our research the priority in ethical approval processing taking into consideration that this study address the hot topic COVID-19 pandemic.

Funding

This research did not receive any specific grant from funding agencies

Conflict of interest

None to declare

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تصور الجمهور لدور الصيدلاني خلال تفشي مرض كوفيد -19 في الأردن

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ملخص

الخلفية: تتطلب جائحة كوفيد-19 عملًا جماعيًا تعاونيًا من قبل جميع المتخصصين في الرعاية الصحية بما في ذلك الصيادلة الذين يمكنهم المساعدة في مكافحة الأمراض الوبائية من خلال تقديم العديد من الخدمات الصيدلانية. ومع ذلك، فإن التصور العام لدور الصيدلي في تقديم الخدمة الصحية مثير للجدل .

الطرق: تم استخدام مسح مستعرض موثوق على شبكة الإنترنت مكون من 25 عنصرًا لاستكشاف رأي المرضى حول قدرة الصيدلي على تقديم خدمات صحية مختلفة أثناء جائحة كوفيد-19. تم إجراء تحليل عامل الاستكشاف لتقييم أفضل نموذج للاستبيان. تم تقييم الارتباط بين المتغيرات الديموغرافية المختلفة والوعي حول دور الصيدلي باستخدام ارتباط بيرسون واختبار مان-ويتني وتحليل التباين أحادي الاتجاه كروسكال واليس.

النتائج: شارك في الدراسة 668 شخصًا. كان متوسط درجات استبيان المستجيبين 97.1 (12.6) وكانت الدرجة القصوى الممكنة 115 (12.9). ارتبط ارتفاع درجة الوعي بزيادة العمر، وجنس الإناث، وانخفاض المستوى التعليمي، والعيش خارج العاصمة عمان، أو كونك طالبًا جامعيًا أو جامعيًا أو يعمل في المجال الطبي.

الخلاصة: إن التصور العام الإيجابي تجاه دور الصيدلي الموضح في الدراسة الحالية ينير الحاجة إلى توسيع دور الصيدلي ليكون أكثر انخراطًا في تقديم الخدمات الصحية المختلفة أثناء الكارثة أو الظروف العادية.

الكلمات الدالة: كوفيد-19، الجمهور، التصور، الصيدلي، التوعية، الخدمات الصحية، الأردن.

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تاريخ استلام البحث 2021/6/7 وتاريخ قبوله للنشر 2022/1/20.

Knowledge, Attitudes and Practice toward Antibiotic Use among Under and Post-Graduate Students at Yarmouk University in Jordan: A Descriptive Study

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ABSTRACT

Objective: Irrational and overzealous use of antibiotics in addition to multidrug resistance has increased at an alarming limits around the world. This study aimed to evaluate the knowledge and practices toward antibiotic use among students at Yarmouk University

Material and Method: A cross-sectional descriptive survey using a structured electronic version of valid questionnaire was distributed among participants. Google form was prepared based on available questionnaire in literature, revised by a group of academic pharmacist to validate the questionnaire. Test-retest was performed for a small group and cronbach alpha was calculated. The form was distributed randomly among under- and postgraduate students at Yarmouk University via their mails.

Results: A total of 1154 individuals who completed questionnaires were analyzed. The knowledge of antibiotic use and resistance was quite good; 72.7% reported correct responses with a mean score of 16 out of 22. High rates of antibiotic use were found with 92% of respondents used antibiotics in the past three months. Inappropriate practice toward antibiotic was common; 48% used it for incorrect indication (e.g., common cold, fever, and pain), 60% used for improper duration and 20% don't take the correct doses.

Conclusions: Interventions on enhancing awareness and understanding of rational antimicrobial use are highly recommended by promoting expert-driven behavioural change, effective communication, education and training. Furthermore, law restriction on antibiotic dispensing should be introduced.

Keywords: Antibiotic, Attitude, awareness, Jordan, rational use.

INTRODUCTION

Overzealous prescription and pharmacy-based dispensing of antibiotics without a prescription are considered a major problem worldwide especially in developing countries [1, 2]. The global problem of antibiotic abuse is owed to various factors including a defect in applying regulations that control dispensing antibiotics, easy accessibility of antibiotics without a prescription from pharmacies, and availability of cheap alternatives in the pharmaceutical market [3, 4]. Additionally, health policies with regard to medical insurance and lack of physicians' concerns about long term resistance may explain the irrational use of antibiotics [5, 6,]. Social and educational factors such as lower educational status, lower knowledge of antibiotics could also contribute to the irrational use of antibiotics [7]. Besides, hazards of untoward reactions and the economic overload on the national health system, overuse of

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Received: 7/10/2021 Accepted: 20/1/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.411*

antibiotics potentially will lead to the growth of resistant bacterial strains and the creation of even multidrug-resistant bacteria [8].

Previously, many studies investigated different patterns of irrational use of antibiotics [9, 10]. A study by Al-Azzam and his colleagues reported a high prevalence of non-prescription use of antibiotics in Jordan [11]. Other studies mentioned that regulation of antibiotic prescription by general practitioners is lacking [12, 13]. Recently, Jarab and his colleagues (2018) observed a shortage in the implementation of guidelines for antibiotic use [14]. A systematic review by Sajal, et al. (2018) mentioned Budwall's note that pharmacists should have an active role in improving antibiotic prescription practice by general practitioners [15, 16]. Similarly, regulatory laws that prevent the un-prescribed dispensing of antibiotics to the adult are present but are not implemented in community pharmacies, which defy the purpose of the legislation of such laws [17].

Antibiotic resistance is a deleterious worldwide problem accelerated by the misuse and overuse of antibiotics, as well as poor infection prevention and control. It is considered a serious global threat [18]. Steps can be taken at all levels of society to reduce the impact and limit the spread of resistance [19]. The general public can help by taking actions such as using antibiotics when prescribed by a certified health professional, always taking the full prescription, never using left-over antibiotics, and never sharing antibiotics with others. Prescribers also should respond to people's expectations and demands, so increasing everyone's understanding of when antibiotics may be of benefit, and when not, should decrease the frequency that they are offered.

Relatively, little is known about the general public's knowledge of antibiotic resistance in Jordan. A previous pilot study [20] was conducted in Amman, and due to the difference in the demographic structure between Amman and Irbid city, the results of this study could not be generalized to the population of Jordan. As complementary to their work, this study could be helpful to gather more information from the northern part of Jordan (Irbid City).

Yarmouk University is a non-profit public higher-education institution located in a large city of Irbid with enrollment range: 40,000-42,999 students from all regions of Jordan. Yarmouk University (YU) offers courses and programs leading to officially recognized higher education degrees such as bachelor degrees, master degrees, doctorate degrees in several areas of study. This study aimed to evaluate the knowledge and practice of antibiotics use, convey health-related information, and evaluate awareness of antibiotic use and bacterial resistance.

MATERIAL AND METHODS

Study design & Setting

A descriptive study was conducted using a pretested, pre-validated structured, and the anonymous questionnaire. The sample size was assessed based on the reports from woldometer.info website: (https://www .worldometers.info/ world-population/jordan-population), which mentioned that the population in northern Jordan is about 2 million and also according to previous studies reporting that the prevalence of antibiotic self-medication may range from 27% to 91.7%. The α-level was set at 5% so that we had a 95% confidence interval (CI) [21]. A preliminary test was elaborated on a 45-participant representative sample (about 4% of the target sample) to address any questions' ambiguity and to determine if the data would provide reliable information. Participants of the pilot study provided the researcher with any feedback they had about the questionnaire items. The feedbacks were considered thoroughly in preparing the final version of the questionnaire that was reviewed by a committee consists of three academic pharmacist and a physician. To be sure that the sample of the present study is random and generalizable, the form has been shared randomly to the entirety of Yarmouk University's students mailing list, which includes all of the University's students. Students from medical and pharmacy colleges were excluded from the final analysis.

Instrument

In this research, a professional electronic version of a valid Arabic questionnaire; that was approved to be valid among the Jordan community, was distributed among participants in Northern Jordan. Shehadeh et al., [20] have developed a questionnaire by reviewing available questionnaires in the literature [22-24]. By then, they translated it to Arabic, tested its content validity and construct validity, and an acceptable Cranach's alpha was found. To be valid for use, the questionnaire was shortened to 22 items in order to make it easier to fill out and increase the number of full responses.

The questionnaire covered the three key points:

1. Knowledge regarding the indications and correct antibiotic use: the purpose of using antibiotics for bacterial or viral diseases; as common cold and infections, when to take it regarding food, duration, what do you do if you miss a dose, etc.
2. Resistance due to misuse: Causes of antibiotic resistance (unnecessary use, not completing the course, no physician's prescription (e.g. self-medication; over-the-counter OTC), using antibiotics with other drugs.
3. Safety: Antibiotic side effects such as allergies.

To enhance the respondents' knowledge and correct their misconceptions, an infographic designed by the World Health Organization (WHO) through visuals using WHO infographic, was shown at the end of the survey. Infographics are embraced because they can rapidly grab attention, simplify complex concepts, and connect components of complex concepts [25, 26] This may help to develop proper interventional programs to improve the public knowledge of antibiotics use, and hence, take a step towards controlling antibiotics resistance.

Statistics

The present work is considered a purely descriptive cross-sectional study. It described the sample characteristics and involved the rate of respondents to each question of the 22-item questionnaire to assess their knowledge and awareness about the use of antibiotics. . Frequency distributions and descriptive criteria were examined. Means for continuous variables and percentages for categorical variables were computed. The knowledge with regard to the purpose for using antibiotics, resistance, and safety was assessed by calculating the number of correct responses out of 22 items. Poor knowledge: scores of 1 (<50% correct response), adequate knowledge: scores of 2 (50-70% correct response), and good knowledge: scores of 3 (>70% correct response) [20]. The reliability of the questionnaire was assessed by calculating the alpha Cronbach's coefficient, which was found to be satisfactory (alpha-Cronbach = 0.71).

Ethical issues

Ethical approval was granted from Institutional Review Board- at Yarmouk University with number (IRB/2021/43)

RESULTS

A total of 1154 respondents matched the the inclusion criteria of the research successfully completed the questionnaire. The mean age for all particpinat (± SD) was 24.6 ±2.3, with 89.4% aged between 18–25 years and mostly were female (791, 69%). Table 1 presents the demographic characteristics of the study participants.

Table 1: Demographic characteristics of the participants

Characteristics	N (%)
Gender	
Female	791 (68.6)
Male	363 (31.4)
Age	

Characteristics	N (%)
18-25	1032 (89.4)
26-35	99 (8.6)
>35	23 (2)
Education	
Undergraduate	1037 (89.9)
Postgraduate	117(10.1)
Marital status	
Single	1048 (90.8)
Married	106 (9.2)

The knowledge score of antibiotic use and resistance of all respondents are quite moderate; 72.7% reported correct responses with a mean score of 2 (adequate knowledge) (Table 2). The results revealed that only half (51.6%) of the sample knew that antibiotics are used for bacterial infections like tonsillitis and tooth infection, 79.9% of respondents knew what dose should they use, with half of them (47.8%) read the dose from internal leaflet, 82.2% aware that antibiotics should be ingested with water not by other drinks as tea or coffee however, about 60.8% did not know what should be the correct duration for antibiotic use. Knowledge about antibiotics resistant was quit good with three quarters (76.9%) knew something about it and 66.2% stated that resistance may be due to using antibiotics for unsuitable un infective diseases, taking improper doses, or using them for the improper duration.

Table 2: Section 1 responses regarding knowledge of participants about how they used the antibiotics

Questionnaire Items	Response	N (%)
indications of antibiotics	Tonsillitis or tooth infection (bacterial infection)	596 (51.6)
	Headache	85 (7.4)
	Fever	126 (10.9)
	Flu/common cold	232 (20.1)
	Muscle pain	35 (3)
	Don't Know	80 (7)
Know the exact dose of antibiotic you should take	Yes	922 (79.9)
	No	232 (20.1)
The source from which you know the dose	Internal leaflet	552 (47.8)
	Pharmacist	311 (26.9)
	Previous experience	185 (16)
	Education level (background of medical knowledge)	106 (9.3)
Know the correct duration of antibiotic use as prescribed	Yes	452 (39.2)
	No	702 (60.8)
If the infection recurs again after some	Yes	848 (73.5)

Questionnaire Items	Response	N (%)
Time, do you think it will be effective again?	No	306 (26.5)
Know about the storage condition of drugs as shown in the leaflet	Yes	882 (76.4)
	No	272 (23.6)
Do you know how the antibiotic can be ingested orally?	Direct without a drink	150 (13)
	Ingested with water	871 (75.5)
	Ingested with coffee or tea	133 (11.5)
Do you know if repeated use of antibiotics is dangerous or not?	Yes	948 (82.15)
	No	206 (17.85)
Do you know why the antibiotic may not produce a response?	When it is not necessary to use it*	146 (12.6)
	Not taking the full course of antibiotic	200 (17.3)
	Self-medication*	357 (31)
	Administering it prior to meals	120 (10.4)
	Using it in cases of fever *	56 (4.8)
	Taking many drugs at the same time*	163 (14.2)
	Using an alternative (same antibiotic but different company)	112 (9.7)
Do you know something about resistance to an antibiotic?	Yes*	887 (76.9)
	No	267 (23.1)
Based on your knowledge, what makes bacteria resist antibiotics?	Using unsuitable antibiotic for the disease*	95 (8.23)
	Using improper dose	82 (7.12)
	Using antibiotic for improper duration*	213 (18.45)
	All previous causes	764 (66.2)
* Statement used in scoring respondents' knowledge.		

Data on participants' behavior and attitude toward antibiotic use are shown in Table 3. Results revealed overzealous use of antibiotics as it showed that 1063 (92%) of the respondents had used antibiotics in the past three months with only 70.1% were prescribed by a physician to treat the condition. Good adhrenec to antibiotics was revealed by 81.5% took the exact doses as prescribed by the physician or pharmacist. Regarding the risk associated with antibiotics use, majority of respondents (92.1%) were aware of the side effects associated with taking antibiotics (Table 4).

Table 3: Section 2 responses regarding participants practice toward the use of antibiotics

Questionnaire Items	Response	N (%)
Did you use antibiotics in the past 3 months?	Yes	1063 (92)
	No	91 (8)
How they get their antibiotic	Prescribed by physician	809 (70.1)
	Buy from the pharmacy on the advice of someone other than a doctor	302 (26.2)
	Used old antibiotics	43 (3.7)
Do you use the antibiotic as your physician recommends?	Yes	836 (72.4)
	No	318 (27.6)
Use of antibiotics to the correct duration	Continue to take the antibiotic even if feeling better	646 (56)
	Stop taking the antibiotic when feeling better	508 (44)
Take antibiotic with the exact doses as prescribed by the physician/pharmacist	yes	940 (81.5)
	no	214 (18.5)
If they miss one dose?	Take it once they remember	818 (70.9)
	Take double dose	20 (1.7)
	Skip it	316 (27.4)
Can you use antibiotics for viral infection?	yes	633 (54.9)
	no	521 (45.1)
After the use of antibiotics, what about the persistence of symptoms?	Disappear	146 (12.7)
	Decrease	266 (23)
	Not disappear and visit a physician	742 (64.3)
After the improvement of an infection, if symptoms recur again, what do you do?	Visit pharmacist and take the same antibiotic	613 (53.1)
	Visit the physician to take his/her advice	122 (10.6)
	Increase the dose by myself	89 (7.7)
	Stop using the previous one and use a new antibiotic by myself	330 (28.6)
Do you use antibiotics before meals?	Yes	957 (82.9)
	No	197 (17.1)
Do you follow the physician's recommendations?	Yes follow physician's recommendations	390 (33.8)
	No, stop its use without taking physician's advice	228 (19.7)
	No, decrease the antibiotic dose without taking physician's advise	246 (21.4)
	Not using antibiotics regularly?	290 (25.1)

Table 4: Section 3 positive response of participants about the risk associated with antibiotics use

Danger mentioned	N (%)
Antibiotic side effect / Allergies	1063 (92.1)
The emergence of drug resistance	806 (70)
Overdose of antibiotics	654 (56.7)
Know there are dangers but don't know what they are	264 (22.8)

DISCUSSION

Antibiotics remain the first therapeutic option to many communities worldwide [27]. Hence, it is essential to assure quality and safe drug use by regulating issues such as self-medication, which is an important determinant of improper use of antibiotics [28]. Previous study was conducted among pharmacy student to evaluate the practice of self-medication among pharmacy students in the University of Jordan, and the prevalence of self-medication among them was 86.7%, and antibiotics was one of the most commonly used drug. [29] The present study was the first to assess the current knowledge and practices in the consumption of antibiotics among young age group mainly at Yarmouk University. The general knowledge of antibiotic use and resistance is quite moderate; 72.7% of respondents reported correct responses with a mean score of 16 out of 22. On contrary, a previous pilot study conducted in Amman [20] showed that the knowledge of antibiotic use and resistance is inadequate in Jordan with <50% reported correct responses. Not surprisingly, as our study differs from the aforementioned work in sample size and the percentage of unawareness of respondents.

Surprisingly, high rates of antibiotic use were found. In fact, 92% of respondents (>18 years old) reported having used antibiotics in the past three months. Results of the present study are significant higher than a recent study in Jordan by Yusef et al., [30] who reported that 41% of the sample, have received oral antibiotics in the past two months, of which 38% acquired antibiotics without a prescription.

In addition, the majority (70%) stated that the antibiotic was prescribed by the physician to treat the condition while one-quarter of individuals (29%) had followed the advice of someone other than the physician or had used an old prescription before taking the antibiotic. These findings were better than those reported in a survey in Kuwait [31] where only 43% were prescribed antibiotics by a physician to treat the condition, while 57% used an old prescription or took someone else's advice. This clearly indicates how much the laws that control the sale of antibiotics in pharmacies in the Jordan republic are followed comparing to Kuwait. Literature confirms that such practices could increase the development of resistant strains of microorganisms [32]. Similarly, some studies have shown that in developing countries, antimicrobials may be obtained without prescriptions from qualified medical personnel, even though the drug regulatory agencies in the countries designate these medicines as prescription-only [33]. In Iraq, testing the bacterial isolates from urine, blood, throat swab, sputum and purulent wounds samples collected from Baghdad hospitals which included the following species of bacteria: GAβHS, K. pneumoniae, S. aureus, Proteus spp and Ps. Aeruginosa revealed that the above bacteria have developed mostly a high degree of multi-drug resistance.[34] To control the emergence of of multi-drug resistance to antimicrobials there is an urgent need to formulate a policy to reduce the inappropriate antibiotic use.

Antimicrobial agents are the most commonly used and misused among all drugs and the consequences of the wide spread use of antimicrobial drugs has increased the emergence of antibiotic resistant pathogens which necessitate the need for new drugs (16-21).

Regarding the indication of antibiotics, the study populations were less knowledgeable pertaining to the indication of antibiotics for the treatment of viral infections. The proportion of respondents who thought that antibiotics are effective for viral infections was comparable with a survey conducted in Britain, Europe, Denver, Wisconsin, and Minnesota (54-55%) [35-38], but better than proportions reported from New Jersey (70%) [39] and Malaysia (67%) [40]. The possible reason for the inadequacy of knowledge in this area could be due to the term "germ", which was normally used during counseling or provision of medical advice to the public/patients instead of using the microbiological term "bacteria" or "virus".

Interestingly, 92% of the participants said they were aware of the risk associated with antibiotic use. Among those aware of the risks, only 70% mentioned the emergence of drug resistance as a consequence as antibiotic use, while 23% said that they knew there were dangers in taking antibiotics but they did not know what they were. These results demonstrate an evident lack of knowledge and poor practice towards antibiotic consumption.

The most important issue regarding the resistant causes is the duration of the antibiotic course. Only about half of the respondents (56%) had correct knowledge of the need to complete the full course of antibiotics when symptoms of infection are improving. A similar proportion of respondents with correct knowledge were noted in the current study when compared with other studies done in Syria (50%) [7], Hong Kong (58%) [24], and Taiwan (50.1%) [23].

The present work revealed a lack of knowledge with the use of antibiotics in the northern part of Jordan as evidenced by the high percentages of those who know that antibiotics can be used when it is not necessary to use them, those who self-medicating themselves with antibiotics, those who ingest them in cases of fever without knowing its origin and if it is secondary to bacterial or viral infections, those who take many drugs concomitant with antibiotics allowing for potential drug interactions, those who take an unsuitable antibiotic for the infective disease in addition to those who improperly use the antibiotics either using improper dose or using it for the improper duration. Other previous studies in Arabian countries are in the same line of bad knowledge of their population about antibiotic use. A study by Tshokey et al. (2017) found also unsatisfactory knowledge and practices toward antibiotic use [41]. On the same line, more than one-third of the Kuwait population did not complete the prescribed antibiotic course and some had self-medicated with antibiotics [42].

Overall, the assessment of knowledge on antibiotic consumption reveals several risk factors associated with irrational use of antibiotics; consumption of antibiotics for common cold and fever, lack of knowledge on the accurate dose and frequency, and poor compliance to antibiotic therapy, which is indirectly indicated by the lack of knowledge on the duration of the antibiotic course. Gualano and his colleagues mentioned that about one-third of the population in low and middle-income countries lack knowledge about antibiotics [43]. These factors are known to contribute to the irrational use of antibiotics and the development of resistance [27]. Interventions on enhancing awareness about antibiotics and resistance and introducing standard therapeutic guidelines should be conducted.

LIMATATIONS OF THE STUDY

Some limitations of this study are that only one university in Jordan was examined; a multi-institution study may reveal higher rates of student participation. In addition, the snapshot nature of the cross-sectional design restricts the generalizability of the study outcomes. Furthermore, some of questions' responses may exposed to subjective variability and reported bias.

CONCLUSION AND FUTURE PERSPECTIVE

Despite that the knowledge of antibiotic use and resistance among Yarmouk University's students was quite good; they have a defect in following the instructions

regarding the proper doses and duration of treatment. Interventions to enhance their awareness about rational usage of antibiotics are highly recommended by promoting expert-driven behavioural change, education and training.

Authors Contribution

LMM had a major contribution in designing and coordinating the study, she conceived the research idea, participated statistical analysis, and interpretation of data, and drafted the manuscript. DA conceived the research idea and helped in editing the final version of the manuscript for publication. KMM participated in statistical analysis and interpretation of data HAK and RT contributed to writing, revising, and editing the manuscript and designing figures. LAI and MAN are medical students at Yarmouk University in Jordan. They helped in the interpretation of data, drafting, and editing the final version.

Conflicts of interest

The authors declare no conflict of interest

Funding

This work was not supported by any funds.

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المعرفة والمواقف والممارسة تجاه استخدام المضادات الحيوية بين طلاب البكالوريوس والدراسات العليا في جامعة اليرموك في الأردن: دراسة وصفية

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ملخص

الهدف: زاد الاستخدام غير العقلاني والمفرط للمضادات الحيوية بالإضافة إلى مقاومة الأدوية المتعددة في حدود تنذر بالخطر في جميع أنحاء العالم. هدفت هذه الدراسة إلى تقييم المعرفة والممارسات تجاه استخدام المضادات الحيوية لدى طلاب جامعة اليرموك

المنهج البحثي: تم توزيع استبانة للمسح المقطعي باستخدام نسخة إلكترونية منظمة من استبيان صالح على المشاركين. تم إعداد نموذج بناءً على الاستبيان المتاح على google form، والذي تمت مراجعته من قبل مجموعة من الصيادلة الأكاديمية للتحقق من صحة الاستبيان. تم إجراء اختبار test-retest وتم حساب .Cronbach alpha تم توزيع الاستمارة بشكل عشوائي على طلاب البكالوريوس والدراسات العليا في جامعة اليرموك عبر البريد الإلكتروني.

النتائج: تم تحليل ما مجموعه 1154 فردًا ممن أكملوا الاستبيانات. كانت المعرفة باستخدام المضادات الحيوية ومقاومتها جيدة جدًا؛ حيث بلغت 72.7٪ عن استجابات صحيحة بمتوسط درجة 16 من 22. وقد كانت نسبة استخدام المضادات الحيوية عالية حيث أنه 92٪ من المستجيبين استخدموا المضادات الحيوية في الأشهر الثلاثة الماضية. كانت الممارسة الخاطئة تجاه المضادات الحيوية شائعة؛ حيث تم استخدامه 48٪ لدواعي غير صحيحة (على سبيل المثال، نزلات البرد والحمى والألم)، واستخدم 60٪ لمدة غير مناسبة و 20٪ لم يأخذوا الجرعات الصحيحة.

الاستنتاجات: توصى هذه الدراسة بشدة باتخاذ الإجراءات اللازمة لتعزيز الوعي والفهم للاستخدام الرشيد لمضادات الميكروبات من خلال تعزيز التغيير السلوكي بالاستعانة بالخبراء والتواصل الفعال والتعليم والتدريب. علاوة على ذلك، يجب إدخال قيود قانونية على صرف المضادات الحيوية. من غير وصفة طبية.

الكلمات الدالة: مضاد حيوي، موقف سلوك، وعي، الأردن، استخدام عقلاني.

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تاريخ استلام البحث 2021/10/7 وتاريخ قبوله للنشر 2022/1/20.

Exploring the Economic Aspects of β-Thalassemia in Jordan in 2019

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ABSTRACT

Thalassemia are inherited hematological disorders considered among the most common genetic disorders worldwide, occurring more frequently in the Mediterranean Region. The WHO estimates that Beta-thalassemia affects 2.9% of the world's population. In Jordan, the carrier prevalence rate of thalassemia is from 2-4%. Patients with thalassemia need a lifelong care, devastating their quality of life and imposing overwhelming psychological and financial burden on patients and their families. The Jordanian Ministry of Health (MOH) is the sole facility responsible for treating these patients from the pre-marital program until required medications regardless of their nationality. This study aimed to estimate the economic burden of thalassemia in Jordan in 2019. All 680 thalassemia patients admitted to thalassemia centers in Jordan and coming to out-patients' clinics from July 1st to Aug 31st, 2019 are included. Data were collected using a pre-developed questionnaire from the electronic medical records. The economic burden was estimated from MOH perspective and societal perspective. The average annual cost was estimated to be 2,674 JOD for a single thalassemia Jordanian insured patient and 4,627 JOD for un-insured, while the non-Jordanian patient' annual cost was estimated 4,751 JOD if insured and 6,651 JOD if un-insured. The total economic burden of thalassemia in Jordan in 2019 was estimated to be 2,148,741 JOD. Of this amount, 1,393,329 JOD was for Jordanians and 755,412 JOD for non-Jordanians. In conclusion, this high burden of thalassemia in Jordan requires adopting new controlling policies; pre-marriage counseling, education and raising awareness should be encouraged.

Keywords: Thalassemia, Jordan, economic burden, 2019.

INTRODUCTION

Jordan is in the western Asia part of the Middle East in an area of political instability. Jordan is an upper middle-income country, with a population of 10.554 million (4.966 million females=47.1% and 5.588 million males=52.9%), Average annual live births is 197,280 (2019). The Gross Domestic Product (GDP) amounted to 31.435 billion JODs ($ US$44.4 billion), and Jordan GDP Per Capita reached 2,990 JOD ($4,222) in 2019. Jordan has a small economy with limited natural resources (1).

The total expenditure on health in Jordan amounted to 2.566 billion JOD ($ 3.6 billion), and the per capita

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Received: 14/11/2021 Accepted: 24/1/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.412*

expenditures was 255 JOD ($ 361) in 2017 accounting for 8.9 percent of the GDP which is considered high for an upper middle-income country. Expenditures on pharmaceuticals was very high and reached 593 million JOD (US $ 838 million) in 2017 accounted for 2.05 percent of the GDP and 23.13 percent of the total health expenditures. Public expenditures on curative care accounted for 73.7 percent while expenditure on primary care accounted for 19.6 percent in 2017 (2).

The health sector in Jordan is heavily subdivided to multiple health providers including public, private, international and charity sectors. The largest provider of health care is the public sector via the ministry of health MOH, providing insurance to 40% of the population, followed by the Royal Medical Services (RMS), covering 27.5% of the population. In addition, Jordan University Hospital JUH and King Abdullah University Hospital (KAUH) provide health care services for the Universities employees and dependents and also serve as referral centers (3).

Most of the leading hospitals in Jordan are accredited by the Joint Commission International (JCI) and/or the National Health Care Accreditation Council (HCAC) (4).

Many rare diseases cause chronic or progressive physical deterioration, disability, or premature death and start in childhood, creating a huge burden on parents and caregivers. Most rare diseases are thought to be genetic. Although there is increasing demand for therapies for rare diseases, drug companies were not interested in adopting them to develop treatments, and as such became known as orphan diseases. The development of new rare disease therapies has encountered significant obstacles with respect to understanding the incidence and prevalence (epidemiology), patient reported burden of disease, economic cost of the disease and treatment, health technology assessment, and patient access (5).

There is no universal agreed on definition of what constitutes a rare" disease. A recent survey of definitions from more than 1,100 organizations worldwide found significant variation, ranging from prevalence thresholds of five to 76 cases per 100,000 population (6). Individual rare diseases affect less than 5 to 7 individuals in 10,000 (7).

β-Thalassemia is a single-gene inherited haemoglobinopathy characterized by a decreased production of globin chains, resulting in chronic anemia and skeletal and organ deformities. The World Health Organization (WHO) estimates that β-thalassemia affects 2.9% of the world's population, creating a major public health problem that burdens health care systems and significantly impacts the quality of life of the affected patients 2011 (8). The survival of individuals with β-thalassemia major is reliant on monthly blood transfusions and iron chelation therapy (9). Without blood transfusions, death usually occurs within the first few years of life. The average life expectancy of those with β-thalassemia major is 32 years compared to more than 75 years general life expectancy, and much shorter if untreated (10) . Additionally, regular blood transfusions cause iron overload, leading to progressive cardiac damage and death. The annual cost of blood transfusions for β-thalassemia major patients in the Middle East has been estimated at USD 3,200 per patient (2015) (11-15).

The prevalence of β-thalassemia in the Middle East is high, where 1-15% of the population carries the trait (12, 15). A major contributor to the high β-thalassemia prevalence in the Middle East is the high prevalence (25-60%) of consanguineous marriages, particularly among first cousins (16, 17). Due to the high burden β-thalassemia places on patients, families and health care systems in the Middle East, the WHO advocates prevention and reduction of the burden of β-thalassemia through voluntary genetic screening (18). In poor countries, high costs of treatment and the lack of receiving adequate measures of healthcare cause death to many thalassemia children and adolescents (19-21).

Timely blood transfusion appears to prevent early signs and symptoms of the disease, however, transfusion complication such as excess iron is deposited in all body organs leading to heart failure (22, 23), chronic liver diseases, endocrine problems, growth disorders,

osteoporosis (24-26) …. etc. The latter leads to increased mortality in these patients (27). Thus, health management as well as planning the required services for early diagnosis and patients' treatment is essential (28-30).

Today, the life expectancy of patients with major thalassemia has significantly increased along with therapeutic advances, and this has changed thalassemia from a fatal to a chronic disease (27). As a result, thalassemia patients need lifelong care, but this requires high spending on blood transfusion, iron chelation drugs, laboratory tests, treatment of side effects, periodic visits, and indirect costs such as the costs of lost opportunities as well as lost welfare and decrease in quality of life (31). Since healthcare funders are seeking to control the costs and effectively allocate the resources (32), having knowledge of the invested costs for thalassemia patients is essential for optimal allocation of resources in this sector. The carrier prevalence rate of thalassemia in Jordan is around 2- 4% (17, 33), according to thalassemia department head in MoH, number of registered cases with B-Thalassemia are around 1450 patients, in which almost 1228 Thalassemia patients are treated.

The aims of the study were to estimate the actual economic burden of β-thalassemia in Jordan in 2019 from the perspective of the payer (Ministry of Health-MOH) (Direct costs), and to inform healthcare policy makers toward better decisions help in containing the economic burden of thalassemia in Jordan.

Methodology:

This study is a retrospective descriptive study. In order to achieve the objectives of the study, a research team was formed consisting of: Physicians from the following departments in MOH: Thalassemia and Hemophilia, Prevention of Genetics Disorders, Non-Communicable Diseases, Cancer Control, Cardiovascular Prevention, Primary Healthcare and a health economist with the following main duties: supervising closely data collection from the 4 thalassemia treatment centers (Middle, North, & South) in Jordan, classifying thalassemia patients in Jordan demographically and clinically, identify, measure and evaluate costs of managing thalassemia in Jordan and estimating them, quantification of the economic burden of thalassemia in Jordan from the perspective of MOH and reporting the results, drawing conclusions and make recommendations.

Data collection was performed by filling a questionnaire specially designed for the study based on a literature review, revised, and approved by the research team experts after a group discussion meeting. Ethical approval was obtained from MOH IRB committee.

Study population is composed of all patients came to the hospitals and thalassemia outpatient's clinics from (July 1, 2020 – Aug 31, 2020); and/or admitted to the 4 thalassemia centers in Jordan (AL Bashir and Zarga hospitals in the middle region, Rahmeh hospital in the north and Al-Ghor hospital in the south). For these patients, registry medical files were studied from (Jan 1, 2019 to Dec 31, 2019 i.e. for 12 months). The total number of patients was 680 representing 55% of all registered thalassemia patients in Jordan.

Twelve Doctors, 2 registered nurses, 3 data entry employees and a facilitator collected the required data. Jordanians, non-Jordanians, all age groups and both sexes were included in this study.Data were obtained from electronic medical records (patients' medical files). Due to lack of electronic data in Al-Ghor, data was obtained manually from the patients' medical files. In addition, interviews with patients /caregivers were made to complete any missed data.

It is worth mentioning that thalassemia treatment protocols in Jordan are according to the Thalassemia International Federation treatment protocol (TIF).

Costs' data were obtained from the accounting department of the MOH in Jordan for all items included, categorized for Jordanian health insured patients and non-Jordanian patients that are supposed to pay fees for the service. The latter include medications, lab tests and imaging tests. All prices were based on MOH tenders

winning prices in 2019.

Collected data were entered and analyzed using XL 2016.

Results and Discussion:

The study includes a total number of 680 patients (demographics were detailed in Table 1): 398 patients from AL Bashir, 151 patients from Irbid, 91 patients from Al Zarqa and 40 patients from Ghor Al Safi treatment center.

Table (1): Demographic description of thalassemia patients in Jordan (2019) (N=680)

Item	Amman	Irbid	Zarqa	Ghor Safi	Total	%
Total number	398	151	91	40	680	100
Jordanians	275	129	77	40	521	76.6
Non-Jordanians	123	22	14	0	159	23.4
Male	196	87	46	26	355	52.2
Female	202	64	45	14	325	47.8
Average age (Years)	20.14	18.71	18.8	16.55	18.55	
Lowest age (Years)	1.5	1	2	1.8	1	
Highest age (Years)	67	44	57	36	67	

Jordan's geographic location surrounded by unstable countries increases the burden on the healthcare system in Jordan resulting in big need of support from all international donors to provide a good, equal service and treatment to all patients residing in Jordan.

The average age of patients in the study is 18.5 years, this means that the majority is still young either studying or working who need care and good health in order to participate in the country production.

Table 2 shows different types of thalassemia in Jordan where Thalassemia Major is the dominant (81% of the total number of thalassemia patients), most of the cases were in the capital (Amman).

Table (2): Types of Thalassemia in Jordan

Item	Amman	Irbid	Zarqa	Ghor Safi	Total	%
Major Thalassemia	325	137	61	27	550	81
Intermediate Thalassemia	67	14	18	7	106	15.6
Sickle Thalassemia	1	0	11	6	18	2.65
Alpha thalassemia	5	0	1	0	6	0.9

The average age of diagnosis as shown in Table 3 is considered late (about 22 months), this may because thalassemia major, which is usually diagnosed before the age of 12 months, has been combined with the diagnosis of thalassemia intermediate.

It was found that first and second-degree relatives' consanguineous marriage rate is 67% (Table 4), which is considered high compared to the Arabic region, published studies showed that Jordan relatives marriage rate ranges from 30-50% (34-40) from the total general population. As it is one of the recessive diseases that are transmitted from the father and the mother; the marriage of relatives is considered a risk factor for having a child with thalassemia. Therefore, the specialized health authorities in Jordan and all partners working in the (pre-marital screening test program) must focus on trying to convince the local society of the need to decrease inbreeding.

Table (3): Diagnosis age (Months) for thalassemia patients in Jordan

Item	Amman	Irbid	Zarqa	Ghor Safi	All
Average diagnosis age (Months)	30.16	7.69	17.8	31.8	21.86
Lowest diagnosis age	1	2	1	60	1
Highest diagnosis age	564	36	211	108	564

Table (4): Percentage of degree of relativeness of Thalassemia patients' parents (Consanguineous marriage) in Jordan

Item	Amman	Irbid	Zarqa	Ghor Safi	Total	%
Parents not relatives (Not consanguineous marriage)	140	41	30	13	224	33
Parents are relatives (consanguineous marriage)	258	110	61	27	456	67

The average blood units (packed RBCs, washed or filtered) consumed per year for each patient is 23.5 units (i.e. almost 2 units/ month), this is considered good and enough (38, 39, 41).

While the average time of giving blood to patients with thalassemia major is every 21 days, it was found that each thalassemia patient blood transfusion time is increased to be every 31 days (Table 5), the latter may be attributed to the inclusion of thalassemia intermediate and sickle cell thalassemia in this study.

Table (5): Blood transfusion (BT) consumption for all thalassemia patients in Jordan

Item	Amman	Irbid	Zarqa	Ghor Safi	Total
Average BT interval (Days)	37.666	24.41	28.3	33.5	30.96
BT units consumed / year	10,090	3,625	1,621	586	15,922

There are some differences in the level of hemoglobin (Hg) for thalassemia patients before giving blood (pre transfusion) among the 4 areas in Jordan, but this level is not too far from the typical level of Hb according to Thalassemia International Federation (TIF) protocol is (9-10.5) (38, 42) (Table 6).

Table (6): Hemoglobin (Hg) and ferritin levels for thalassemia patients in Jordan

Item	Amman	Irbid	Zarqa	Ghor Safi	Average
Pre BT-Hg level (average) (Last reading in g/dL)	8.75	8.313	7.65	6.3	7.75
Lowest Pre BT-Hg (g/dL)	5	6.2	6.5	5	5.68
Highest Pre BT-Hg (g/dL)	10.5	10.6	11	9	10.6
Average Serum ferritin (ng/dL)	2659	2867.5	3290	1291	2526.9
Lowest Serum ferritin (ng/dL)	40	12	32	75	39.75
Highest Serum ferritin (ng/dL)	12272	9480	12725.5	4300	12725.5

As thalassemia patients in Jordan are usually given an adequate amount of blood, the average Serum ferritin level (2526 ng/dL) is considered high, it should be decreased to around 1000 ng/dL by giving patients chelating agents. The discrepancy between centers of the same may be attributed to different amounts of blood given to the patients in those centers, and patients' adherence to taking iron chelation agents (42).

Blood in Jordan is neither sold nor bought, rather is given to patients free but by bringing a donor, the latter is an exception for thalassemia patients (no need to bring a donor). The results showed that the cost of the blood transfusion accounts for almost 16.5% of the total economic burden of thalassemia in Jordan (Table 7). According to the Jordan National Blood Bank workers and experts, the real cost of each unit of blood in Jordan is 150 JODs compared to more than € 439 (equivalent to 369 JODs) in many European countries (42).

Table (7): Annual cost of blood units consumed for thalassemia patients in Jordan

Thalassemia patients	Units	Unit cost	Total cost **(JODs)**
Able, not insured Jordanian	12,025	15.0	180,375
Non-Jordanian	3,897	45.0	175,365
Grand total			355,740

The total number of patients treated with iron chelation agents in the study were 556, representing 81.7% of all patients (i.e. there are 18.3% not treated). The annual cost of iron chelation drugs according to the preferential prices for the MoH accounts for almost 55% of the total cost of treating thalassemia in Jordan (Table 8). The latter is consistent with international percentages worldwide. It is worth mentioning that the prices of iron chelating drugs in Jordan were decreased during the last 3 years. Besides, the adoption of the same international protocol of TIF in treating thalassemia in the 4 centers enables the MoH to buy large quantities with lower prices (economies of scale) through a unified annual tender, furthermore the availability of low-price generic substitutes manufactured locally also decrease the cost.

Table (8): Cost of iron chelating agents

Chelating agents	**Units**		**Unit Cost [40] (JOD)**		**Total Cost (JOD)**		**Total (JOD)**
	Able, not insured Jordanian	**Non- Jordanian**	**Able, not insured Jordanian**	**Non-Jordanian**	**Able, not insured Jordanian**	**Non-Jordanian**	
Deferasirox 500 mg Tablet	376,810	111,375	1.6	2.4	602,896	267,300	870,196
Deferasirox 250 mg Tablet	29,003	10,175	0.9	1.4	26,102.7	14,245	40,348
Deferasirox 600 mg Tablet	608	103	2.0	2.9	1,216	299	1,515
Deferiprone 500 mg Tablet	69,798	17,979	1.5	2.4	104,697	43,149	147,846
Deferoxamine inj. Vial	17,530	6339	4.0	6.5	70,120	41,204	111,324

Chelating agents	Units		Unit Cost [40] (JOD)		Total Cost (JOD)		Total (JOD)
	Able, not insured Jordanian	Non- Jordanian	Able, not insured Jordanian	Non-Jordanian	Able, not insured Jordanian	Non-Jordanian	
Distilled water	14,280	4,222	0.05	0.1	714	422	1,136
Scalp vein	3,920	1,346	0.3	0.3	1,176	403.8	1,580
Syringe 20 ml	4,234	2,261	0.1	0.1	423.4	226.1	650
Hydroxyurea 500mg Tablet	5,648	1,740	0.2	0.3	1,129.6	522	1,652
Other medications					14,600	37,755	52,355

Not all thalassemia patients in Jordan accept taking seasonal flu vaccine despite their doctors' advice. As for the hepatitis vaccination, it is originally taken with the National Vaccination Program for all newborn babies in the first months of life but is considered here for those discovered with no antibodies, so additional vaccine doses were re-administered. Prevenar 13 vaccine is given to patients undergoing splenectomy, but the cost of this vaccine is mostly paid by the patient. Because of these, the cost of vaccination is considered negligible and not included.

The cost of medications other than chelating drugs represent only 1.85 % of the total cost. The latter means that chelation drug still very expensive (Table 8).

The three important imagining examinations required for thalassemia patients are Abdominal Ultrasound (Abd US), Echo and Dexa scan costs 14,727 JOD annually accounting for 91% of all imaging costs. It is worth mentioning that although MRIT2 imaging is very important to follow the accumulation of iron in the heart and liver for thalassemia patients, it is not included as it is not available in MoH.

The three most important annual routine tests for thalassemia patients according to the international protocol of TIF (43) are: cardiac examination, abdominal examination and the most complicated one (osteoporosis) with very high cost. In order to follow thalassemia patients, it is imperative to conduct many different lab tests. Table 9 shows the costs of these lab tests which accounts for 9.2% of the total cost in which kidney- liver electrolytes, CBC, Vit D, thyroid, TSH, Vit B12 and folate are the major costly ones.

The main complications of thalassemia are (Osteoporosis, Hypothyroidism, Diabetes mellitus, Cardiovascular Disease and Hypogonadism); treating these complications increases the cost of treatment and follow-up for thalassemia patients in outpatient clinics as well as admitted to hospitals.

Table (9): Lab tests cost for thalassemia patients in Jordan

Item	QYT	Unit Cost (JODs)		Total Cost (JODs)		Total (JODs)
Lab tests		Able, not insured Jordanian	Non-Jordanian	Able, not insured Jordanian	Non-Jordanian	
CBC	9962	3.0	6.5	23156.7	14580.2	37736.9
KFT, LFT, S. elec	2005	28.0	52.8	42189.3	26282.2	68471.4
Screening: Hb electrophoresis	680	5.0	7.0	2605.4	1112.5	3717.9
Ferritin	1901	4.0	10.0	5651.0	4882.4	10533.4

Item	QYT	Unit Cost (JODs)		Total Cost (JODs)		Total (JODs)
Lab tests		Able, not insured Jordanian	Non-Jordanian	Able, not insured Jordanian	Non-Jordanian	
TFT	1477	15.0	33.0	16098.7	13323.9	29422.6
Folic acid, B12	916	12.0	20.0	8004.2	4979.7	12983.9
VIT D	978	25.0	30.0	17771.5	8014.2	25785.7
sex Hormone	134	6.0	15.0	694.4	274.0	968.4
HbsAg, HbsAb, HCV Ab	682	9.0	30.0	4941.0	3989.9	8930.9
Others						3822

Osteoporosis accounts for more than 50% of the complications costs which is high when compared to other studies; the latter need further research particularly as TIF advice that all patients more

than 10 years old must undergo bone scanning and be treated by age of 18 (44).

Table (10) shows the length of stay in hospitals costs including emergency visits, outpatient visits, admission, and any operation related to disease complications if any. The latter represents 8.6 % of the total cost, noting that the approved prices by MoH are very much less than those in other hospitals in the private sector, which is estimated to double or triple these numbers.

.Table (10): Length of stay (LoS) in Hospital and outpatients' costs for thalassemia patients in Jordan (JODs)

Item	QYT	Unit Cost (JOD)		Total Cost (JOD)		Total (JOD)
		Able, not insured Jordanian	Non-Jordanian	Jordanian	Non-Jordanian	
Outpatient visits/yr	8135	3.3	8.0	19779.0	17130.9	36909.9
Inpatient visits/yr	2952	14.7	36.0	37140.8	15004.4	52145.3
LoS in Hospital/yr	252	3.0	16.0	591.3	878.6	1469.8
LoS in ICU unit/yr	336	3.0	79.0	699.9	8113.3	8813.2
Emergency visits/yr	495	0.4	8.0	184.6	267.2	451.8
Cholecystmies	113	300.0	425.0	26940.0	9860.0	36800.0
Splenectomies	147	300.0	425.0	32448.0	16507.0	48955.0

As thalassemia patients need continuous care, the absentee from work for the family caregivers as a result of taking care of their thalassemia patients were considered; the latter was calculated based on absent working days and transportation to the thalassemia center. These costs were estimated and calculated based on the information obtained from family members accounting for 7.4 % of the total cost of treating thalassemia patients (Table 11).

Table (11): Other costs related to thalassemia (indirect costs)

Item		Total Cost (JODs)		Total (JODs)
		Able, not insured Jordanian	Non-Jordanian	
Transportation related cost/ yr	103043.0	78930.9	24112.1	**103043.0**
Caregiver lost working days/yr	4148.0			
Average wage/day for caregiver	13.6			
Total cost of caregiver lost working days	56412.8	43212.2	13200.6	**56412.8**
Grand total				**159,455.8**

The average monthly family income for patients who treated in Jordanian Thalassemia centers is approximately 338 JODs (ranging from 53 JODs to 1313 JODs; 53 JOD is for refugee's income).

Average family (caregiver) daily income assuming 25 working days per month = 13.6 JODs (dividing 338 JOD by 25 days). Jordan GDP for 2019 per capita is $9,731.1 (45) (Table 12).

Table (12): Average family income of thalassemia patients in Jordan (JODs)

Item	*Amman	*Irbid	Zarqa	Ghor Safi	Total
Family average monthly income	427	387	307.5	233	338.625
Family lowest monthly income	45	30	90	50	53.75
Family highest monthly income	3000	1005	600	647	1313
Average family (caregiver) daily income (JOD) (25 working days per month)					13.6

It was found that the average annual cost for one thalassemia Jordanian insured patient is 2,674 JOD while it is 4,627 JOD for uninsured ones. On the other hand, non-Jordanian patient annual costs is 4,751 JODs if they are subsidized (supported by MOH) and 6,651 JODs if not. The total annual cost of thalassemia for 2019 in Jordan is approximately 2,148,741 JODs in which Jordanians cost a sum of 1,393,329 JODs while the non-Jordanians cost 755,412 JODs paid by the government (Table 13).

As stated by the UNICEF study conducted earlier in 2017 (47), the cost paid out by the MOH was 3,468,035 JODs indicating the high amount of cost paid for the treatment of non-subsidized thalassemia patients in Jordan.

Table (13): Cost of illness of thalassemia in Jordan for 2019 (JOD)

Economic burden of Thalassemia	Jordanians	Non-Jordanians	Total
Annual estimated cost (subsidized)	**1,393,328.9**	**755,411.81**	**2,148,740.66**
percentage	**65%**	**35%**	**100%**
Annual estimated cost (not subsidized) as per UNICEF study (47)	**2,410,458.9**	**1,057,576.5**	**3,468,035.45**
percentage	**69.5%**	**30.5%**	**100%**
Annual average patient cost (subsidized) (43)	**2674.3356**	**4751.0177**	**3159.91274**
Annual average patient cost (not subsidized) (43)	**4626.6006**	**6651.4247**	**5100.05214**

In general, the average annual cost for thalassemia patients, if subsidized, is about 3,160 JD; while if not subsidized the cost is 5,100 JD.

In summary, the subsidized cost for 680 thalassemia patients is 2,148,740 JODs, subdivided as percentages as follows: cost of iron-chelation drugs (54.6%), blood transfusion (16.5%), laboratory tests (9.2%), visits to clinics, admission to hospital, and operations (8.6%), lost working days and transportation costs (7.4%), other medications (1.85%) and imaging examination (0.7%).

Faced with the rising drug bills, health care organizations have focused on methods of cost containment (46). Life expectancy of patients with major thalassemia has significantly increased recently along with advanced new therapeutic treatment protocols. The latter increases the economic burden on healthcare systems as thalassemia patients need lifelong care including: Blood transfusion for life, Iron chelation drugs, Laboratory tests, Treatment of complications and medication side effects, Periodic visits to outpatient clinic and hospitalizations if needed. In addition to opportunities loss and decreased welfare and quality of life.

Longer life expectancy for thalassemia patients who need these healthcare services raises a very important question: How can MOH gives maximum care to patients, contain the cost and try to allocate scarce resources effectively?

Estimating the annual cost for treating thalassemia patients differs from country to country. Some studies in the United Kingdom (34, 48) , Thailand (35) (35), Taiwan(36), Iran (17, 31), USA (43, 44), India (49) (50) estimated the annual costs (2012 through 2015) to treat a thalassemia patient as follows: $18583 in United Kingdom (34, 48), $950 in Thailand (35), $7,464 in Taiwan (36), $2068 and $8321 in Iran (17, 31), $128,062 in USA (43, 44), $629 in India (50). The latter estimated costs variation was attributed to:

1- Different types of medications used to treat of thalassemia and the use of new expensive drugs

2- Different treatment protocols used in each country

3- Differences in hemoglobin level before the next transfusion (pre transfusion Hb)

4- Differences in the level of ferritin if more or less than 1000 at treatment start

5- Patients' compliance to the medication

6- Availability and access to blood transfusion

7- Treatment of complications and follow up

8- Availability of thalassemia treatment centers

9- Year of the study

Prevention programs for eradication of thalassemia have already been applied successfully in Cyprus, Italy (particularly Sardinia), and Greece (51, 52). Another study from the United Arab Emirates has shown that if the carrier rate remains high, carrier-carrier marriages will continue, and it will be difficult to curtail thalassemia major. While thalassemia can be controlled, it cannot be eradicated.

However, countries in the Mediterranean belt and countries such as India, Pakistan, Iran, Turkey, Bangladesh, Sri Lanka, and many others have no alternative but to implement screening programs to bring down the prevalence of thalassemia carrier status (53).

Global migration is another factor for increase in thalassemia cases in countries outside the thalassemia belt. This is one of the reasons why international agencies should come forward to help control thalassemia in the Mediterranean belt (13) (54).

Conclusion:

The Jordanian government spends great amount of money treating thalassemia patients as MOH is the only healthcare body offering this service to all patients regardless if they are Jordanians or not.

It was concluded in this study that one major reason that is considered a high-risk factor for increasing thalassemia in Jordan is the relative marriage, so remarriage investigations must be highly encouraged in this regard. Thalassemia treatment costs are very high and

most of these costs are related to the drugs received by the patients, and most of them are for iron chelation drugs.

Limitations of the study:

- Time of the study was not good enough for data collection and analysis

- It might be difficult to obtain the actual costs values as the study relies on prices given from the accounting department at MOH.

- Being one of the pioneer studies in this field and the lack of previous and similar studies in this scope, made it a little difficult to compare results and verifications.

- Many other costs were not calculated e.g. the cost of the time spent by nurses and doctors, and the cost of other utilities such as electricity, water and maintenance…etc.

- The prices considered by MOH have been greatly reduced compared to international and other national institutions. The latter may be attributed to: giving preferential prices to MOH by drug companies, competition through tendering purchasing process and local manufacturers supplying generics with much lower prices.

Recommendations:

- Increase community awareness and education about the disease and the big impact of consanguinity marriage in spreading thalassemia.

- Further research is needed to study all thalassemia patients in Jordan.

- Improve filing system with proper and sufficient data log keeping with easy and secure access to the data legitimately and may be electronically.

- More training and preparedness for those who accomplish these tasks, in a more scientific and appropriate manner.

Funding: This work was supported as an operating grant from UNICEF (United Nations Children's Fund-Jordan)

Conflict of interest statement: The authors have no competing interests.

Acknowledgments:

Authors would like to thank the good efforts performed by the following MDs in collecting data (Maram Alnawaiseh, Aisheh Alesufi, MoHammad Sweiti, Ahmad Azmi, Ahmad Kheiri, Lateefah Maraqa, Tyseer Alsaheb) and data entry clerks (Arab Almasri, Dania Shaher, Amani Alsayed)

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البحث في الجوانب الاقتصادية لمرض ثلاسيميا- بيتا في الأردن لعام 2019

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ملخص

يعد مرض الثلاسيميا من أمراض الدم الوراثية والاختلالات الجينية الأكثر شيوعا في العالم، ويزداد انشارها في منطقة حوض البحر الأبيض المتوسط. تقدر منظمة الصحة العالمية عدد سكان العالم الذين يعانون من الثلاسيميا (بيتا) ب 2.9%. بينما يقدر معدل انتشار الثلاسيميا في الأردن ب(2-4)%. يحتاج مرضى الثلاسيميا إلى عناية خاصة مدى الحياة حيث يعانون من نوعية حياة سيئة، وأعباء مادية واجتماعية مرهقة على المرضى وذويهم. وتعتبر وزارة الصحة الأردنية الجهة الوحيدة المسؤولة عن علاج هؤلاء المرضى، حيث تتكفل بالإجراءات العلاجية والوقائية بدءا من فحوصات ما قبل الزواج، وحتى تغطية الأدوية المستخدمة بغض النظر عن جنسية المرضى. هدفت هذه الدراسة لتقدير العبء الاقتصادي لمرض الثلاسيميا لعام 2019 في الأردن. وتضم (680) مريضا تم إدخالهم لمراكز علاج مرض الثلاسيميا وراجعوا العيادات الخارجية في الفترة ما بين الأول من يوليو وحتى 31 أغسطس عام 2019. تم جمع البيانات باستخدام استبيان مجهز مسبقا وقام الباحثون بتعبئته من ملفات المرضى الطبية المحوسبة، ثم تقدير العبء الاقتصادي لهذا المرض من منظور وزارة الصحة والمنظور المجتمعي. قدرت الكلفة السنوية المتوسطة لمريض الثلاسيميا الأردني المؤمن ب 2674 د.أ بينما تصل كلفة المريض غير المؤمن إلى 4627 د.أ. وتقدر كلفة تغطية مريض الثلاسيميا غير الأردني المؤمن 4751 د.أ بينما لغير المؤمن من نفس الفئة 6651 د.أ. أما الكلفة الإجمالية فتقدر بحوالي 2,148,741 د.أ. تم صرف 1,393,329 د.أ على المرضى الأردنيين بينما تم صرف 755,412 د.أ على المرضى غير الأردنيين في السنة. وفي النهاية فإن العبء الاقتصادي المرتفع لمرض الثلاسيميا في الأردن يحتاج إلى تبني سياسات للسيطرة على المرض مثل تشجيع الاستشارة قبل الزواج، والتعليم والتوعية حول هذا المرض.

الكلمات الدالة: ثلاسيميا، الأردن، العبء الاقتصادي، 2019.

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تاريخ استلام البحث 2021/11/14 وتاريخ قبوله للنشر 2022/1/24.

Jordan Journal of Pharmaceutical Sciences, Volume 15, No. 3, 2022

Extract and Fractions from Soil Bacteria (*Streptomyces canus* ATCC 12647) Possess Antimicrobial and Anti-Oxidative Potential *in vitro*

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ABSTRACT

Streptomyces species are the most prolific producers of antibiotics within the group actinobacteria. The *in-vitro* antimicrobial and antioxidant activities of the methanol (MeOH) extract and vacuum liquid chromatography (VLC) fractions of a soil bacteria *Streptomyces canus* ATCC 12647 were evaluated. Agar well diffusion method was used for the antimicrobial assay, while phosphomolybdate and DPPH radical scavenging methods were used for the antioxidant assay. The antimicrobial assay showed remarkable activities against *Bacillus subtilis, Staphylococcus aureus,* and *Candida albicans*. Also, the extract and fractions showed good *in-vitro* antioxidant activities in both models. Our results showed that extract and VLC fractions from bacterial isolate had good antimicrobial and antioxidant activities.

Keywords: *Streptomyces canus,* Antimicrobial, Antioxidant, Natural products, Infectious diseases.

1. INTRODUCTION

Human population globally has been devastated by the creasing incidence of infectious diseases, which arose from antimicrobial resistance[1,2]. *Streptomyces* species, characterized by the high level of guanine-cytosine content with the ability to produce bioactive secondary metabolibelongongs to the order *actinomycetale* within the class *actinobacteria* and are among the most important species with diverse gene clusters for the biosynthesis of polyketide, peptides and non-ribosomal peptides[3]. *S. canus* has been identified to produce cyclic depsipeptide telomycin, an antibiotic with noteworthy antibacterial activity[4]. This natural peptide antibiotic exhibits potent *in-vitro* inhibitory activity against gram-positive pathogenic bacteria, including penicillin resistant *Staphylococcus aureus* and vancomycin intermediate *Staphylococcus aureus* (VISA), which are causative agents for hard to treat nosocomial infections[4].

Other isolated bioactive metabolites from *Streptomyces canus* include resistomycin, and tetracenomycin. Resistomycin possesses significant *in vivo* antifungal activity against rice blast[5]. It also, exhibit strong antifungal activity against *Valsa mali* and *Magnaporthe grisea* with IC_{50} of 1.1 μg/mL and 3.8 μg/mL respectively[5]. Column chromatographic separation of the fermented broth of *S. canus* strain FIM0916 led to the isolation of two lipopeptide amphomycin and aspartocin with aspartocin D and E possessing gram positive antibacterial activities[6].

Infectious diseases impose a great deal of oxidative stress to the patients, and there is established link that mounting oxidative stress modifies the diseases pathogenesis[7]. Oxidative stress causes some harmful effects in the body like lipid peroxidation and oxidative damage to DNA[7]. It also plays roles in the development of atherosclerosis, diabetes, inflammation, neurodegenerative diseases like Alzheimer, Parkinson's, and

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Received: 16/12/2020 Accepted: 14/2/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.416*

some other physiological diseases like aging[8].

Reactive oxygen species cause cancer[9, 10]. The implication of this link between infectious diseases and oxidative stress diseases is that antioxidant therapy is needed for the treatment of infectious diseases[11]. In view of this, this study proposes to screen the extract and VLC fractions of the soil bacteria; *S. canus* strain ATCC 12647 for their antimicrobial and antioxidant activities. This will provide researchers with good background for full characterization and isolation of antioxidant metabolites from *S. canus* ATCC 12647.

2. Materials and Methods

Materials

Starch soluble (Acros Organics, New Jersey, USA), Sodium nitrite (Alfa Aesar, England), XAD 7HP and XAD 16N (20 – 60 mesh) (Sigma Aldrich, USA), Agar (Formedium LTD, England), Methanol and Acetone (JHD, China), Silica gel, Vitamin C, Instant Ocean (trace element) (Aquarium Systems, Sarrebouurg, France), DPPH (Sigma Aldrich, Germany). Water purified by a Milli-Q purification machine (Millipore Corporation, Bedford, MA, USA) was used for this study.

Instrumentation

Electronic weighing balance (Mettler, Germany), Rotary evaporator (Büchi Rotavapor R-200), Heating mantle (Philip Harris, UK), Water bath (Philip Harris, UK), Vacuum pump, Avanti JXN-26 Centrifuge (Beckman Coulter), Innova 4300 Shaker Incubator with a 2.5 cm orbit diameter (New Brunswick Scientific), Genevac® EZ-2 Plus (Autur Mckay), Laminar flow cabinet (BH-EN 2004), UV/Visible Spectrophotometer (Shimadzu, Japan) and -80ºC Freezer (Forma Scientific).

Test Microorganisms

The test microorganisms were type cultures stocked in the culture maintenance unit of the Department of Microbiology, University of Nigeria, Nsukka. Gram-positive bacteria; *Staphylococcus aureus* (ATCC 9027), *Bacillus subtilis* (ATCC 35021). Gram-negative bacteria; *Salmonella typhi* (MTCC-531) *Escherichia coli* (ATCC 6538P), and fungi such as *Candida albicans* (MTCC-183) and *Aspergillus niger* (MTCC 961) were used. Microorganisms were maintained by weekly sub-culturing and incubation at 37ºC for bacteria and 25ºC for fungi. Twenty-four-hour culture of each text organisms was used for the assay.

Sample Collection

S. canus is a terrestrial actinomycete isolated from soil in the USA. The *S. canus* strain ATCC 12647 was provided by Prof. RJM Goss, School of Chemistry, University of St Andrews, United Kingdom. Mycelial stocks were preserved in 25% glycerol and stored in a refrigerator maintained at - 80ºC until needed for use.

Culturing Procedures

Starter culture

Starter culture of *S. canus* ATCC 12647 strain was grown on 100 mL (2 *x* 50 mL) volumes of M5 media (0.20 g of soluble starch, 0.01 g of $NaNO_2$, 0.05 g of K_2HPO_4, 0.05 g of $MgSO_4$, 0.15 g of agar, 1000 μL trace element, 100 mL of MilliQ water) for 4 days at 28ºC and 180 rpm. The M5 medium was autoclaved for 20 min at 121°C load temperature before use.

Main culture

The main culture of *Streptomyces canus* strain was grown on 10 L (20 *x* 500 mL) volumes of M5 media (20 g of soluble starch, 1 g of $NaNO_2$, 5 g of K_2HPO_4, 5 g of $MgSO_4$, 1000 μL trace element, 1 L of MilliQ water) with agitation for 7 days at 180 rpm in an Innova 4300 shaker incubator.

Extraction and Purification Procedure

After fermentation of the main culture, broth was centrifuged at 8000 rpm for 1 h at a temperature of 4ºC and the supernatant mixed with XAD-7HP and XAD-16N (1:1, 10% *w/v*), agitated continuously for 7 h and filtered using sintered glass funnel. The resin was then washed with 10.0 L MilliQ water, and extracted with methanol (5.0 L). The solvent was removed at a reduced temperature and pressure using a rotary evaporator to yield the extract[12]. The dry extract was purified using vacuum liquid chromatography (VLC). Briefly, the dried extract (10.8 g) was triturated with silica gel (10.0 g) in a mortar and loaded onto a sintered glass Buckner funnel (6 cm *x* 30 cm, ID) attached to a vacuum line and

packed with graded silica gel 60 (0.04-0.063 mm, 230-400 mesh) as adsorbent, then eluted with methanol in acetone gradient (25, 50, 75 and 100%, 1 L each) to yield the VLC fractions (F1-F4). These sub-fractions were subsequently concentrated to remove the solvents and used for the antimicrobial and antioxidant studies[13].

Antimicrobial Screening of Extract and VLC Fractions

To assay the antimicrobial activity of the extract and sub-fractions, well diffusion method was used[14,15]. Bacteria and fungi were seeded uniformly in nutrient agar and incubated for 24 h at 37ºC and 27ºC respectively, and 10 mL of nutrient agar was inoculated with the single colony formed. The culture was incubated in a Laminar flow cabinet for 24 h. After the incubation a 10% of the inoculum was used to inoculate a 0.5% of Muller-Hinton agar which has been cooled down to 40ºC and then transferred into an agar plate with a cork-borer of 6 mm in diameter. The extract and fractions were diluted two-fold (10, 5, 2.5, 1.25 mg/mL) using DMSO and10 µL volumes were loaded onto each disc with ciprofloxacin and fluconazole as positive controls respectively and DMSO as negative control. Agar plates were incubated at 37ºC for bacteria and 27ºC for fungi and the inhibition zone diameter determined after 24 h and 48 h of incubation respectively. A linear plot of square of inhibition zone diameter (IZD^2) against log concentration to base 10 was made and the minimum inhibitory concentration (MIC) values of the samples determined as the zero intercept of the linear regression[16].

Antioxidant Assay

Phosphomolybdate and DPPH radical scavenging activity methods were used for *in-vitro* antioxidant assay of the extract and VLC factions.

DPPH Radical Scavenging Method

Free radical scavenging activity of extract and VLC fractions were determined using 1, 1-diphenyl-2-picryhydrazyl (DPPH) method[17]. Briefly, 0.1 mL solution of DPPH (4.5 mg/100 mL) in methanol was added to 3 mL of different concentrations (10, 20, 30, and 40 mg/mL) of the samples dissolved in methanol in ependorf vials. The mixture was agitated vigorously and incubated at room temperature for 30 min. Then, the absorbance of mixtures was measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu). Control solution was prepared by mixing 3.5 mL methanol and 0.3 mL DPPH radical solution. Ascorbic acid was used as reference antioxidant compound and the experiment was done in triplicate. The percentage inhibition of the DPPH scavenging activity was calculated using the formula below:

$$Percentage\ inhibition = (1 - \frac{A_1}{A_0})\ x\ 100$$

Where: A_0 is the absorbance of the control and A_1 is the absorbance of the test samples.

Total Antioxidant Capacity Assay (TAC) by Phosphomolybdate Method

The total antioxidant capacity assay of extract and VLC fractions was determined by the phosphomolybdate method[18]. Briefly, 0.1 mL aliquot of the various concentrations (10, 20, 30 40, and 50 mg/mL) of the samples were mixed with 1.0 mL of reagent solution (600 mM of H_2SO_4, 28 mM of Na_3PO_4, and 4 mM ammonium molybdate, 1:1:1) in test tubes and incubated in a water bath at 95ºC for 1.5 h, then cooled to room temperature and the absorbance of mixture was determined at 765 nm against a blank containing 1 mL of the reagent solution. Ascorbic acid was used as positive control. The assay was carried out in triplicate and the total antioxidant capacity was calculated using the formula below:

$$Percentage\ TAC = (1 - \frac{A}{A_0})\ x\ 100$$

Where A_0 is the absorbance of the blank; A is the absorbance of the test samples.

Statistical analysis

The results were expressed as mean ± standard deviation (n = 3) and analyzed using descriptive statistics.

3. Results

Antimicrobial screening

All tested samples exhibited good antimicrobial activity against tested micro-organisms. F4 showed prominent activity against four of the tested organisms while F1 and F2 exhibited good inhibitory activity against *C. albicans* and *S. aureus* respectively (Table 1).

Table 1. Minimum Inhibitory Concentrations of the Extract and VLC Fractions

	Minimum Inhibitory Concentrations (MICs) (µg/mL)					
	A. niger	***C. albicans***	***E. coli***	***S. typhi***	***B. subtilis***	***S. aureus***
Extract	985±0.020	740±0.031	1687±0.031	1049±0.011	1388±0.031	1055±0.022
F1	1732±0.011	197±0.014	1442±0.025	1353±0.013	1252±0.012	1426±0.041
F2	1222±0.033	1278±0.031	1297±0.015	1443±0.012	796±0.022	801±0.028
F3	714±0.022	1104±0.023	816±0.023	453±0.023	855±0.011	417±0.022
F4	435±0.031	841±0.042	336±0.032	168±0.014	633±0.015	1886±0.032
CPF	ND	ND	1.040±0.033	1.637±0.213	697±0.033	594±0.026
FCZ	0.380±0.034	0.144±0.031	ND	ND	ND	ND

F1 – F 4 = solvent fractions, CPF = ciprofloxacin, FCZ = fluconazole, ND = Not tested

Antioxidant Assay

The methanol extract and VLC factions of *S. canus* ATCC 12647 showed potent antioxidant activity. The percent antioxidant inhibition obtained from both models were dose dependent. However, the radical scavenging potentials of the extract and fractions were remarkably higher than that of the total antioxidant capacity since DPPH assay s more sensitive than Phosphomolybdate Method (Table 2 & 3).

Table 2. Radical Scavenging Activity (%) of the Extract and VLC Fractions

	% Inhibition of Samples at different Concentration (mg/mL)				
	10	**20**	**30**	**40**	**50**
Extract	36.71±0.03	41.18±0.10	48.55±0.06	64.06±0.96	64.93±0.14
F1	22.00±0.03	31.91±0.08	36.05±0.26	47.19±0.01	55.49±0.09
F2	17.70±0.12	26.67±0.09	37.02±0.06	38.36±0.09	40.37±0.40
F3	35.33±0.02	37.73±0.12	42.64±0.07	47.37±0.04	60.66±0.04
F4	18.32±0.12	23.58±0.08	36.59±0.22	38.21±0.13	47.90±0.10
Ascorbic Acid	48.31±0.21	64.51±0.16	67.71±0.20	70.42±0.23	72.80±0.24

F1 – F4 = solvent fractions

Table 3. Total Antioxidant Capacity (%) of the Extract and VLC Fractions

	% Inhibition of Samples at different Concentration (mg/mL)				
	10	**20**	**30**	**40**	**50**
Extract	5.21±0.04	10.52±0.01	15.89±0.05	20.85±0.07	30.05±0.08
F1	2.46±0.02	7.41±0.02	15.39±0.02	25.42±0.05	31.93±0.04
F2	0.26±0.01	1.02±0.02	6.17±0.02	14.89±0.01	19.10±0.03
F3	3.30±0.20	5.40±0.08	14.10±0.11	25.00±0.17	26.01±0.04
F4	6.52±0.01	13.71±0.02	19.13±0.04	25.38±0.02	34.72±0.03
Ascorbic Acid	9.38±0.04	19.67±0.33	25.82±0.02	32.99±0.01	44.14±0.83

F1 – F4 = solvent fractions

4. Discussion

Antimicrobial Activity

The genus *Streptomyces* are renowned producers of potent bioactive natural metabolites such as antifungals, antivirals, antitumor, antihypertensive, antioxidants, immunosuppressant especially antibiotics[19,20] and the inhibitory potency of their metabolites has been reported[21]. Today, approximately 80% of the antibiotics are gotten from *Streptomyces*[22] with over 50% clinically useful[23]. *Streptomyces* through this capability of producing these diverse chemical scaffolds which confers wide ranges of biological activity have contributed significantly to mankind[24,25]. *S. canus* ATCC 12647 is a prolific member of soil actinobacteria which produces ranges of metabolites, prominent of which is telomycin and its analogues. These metabolites exhibit strong bactericidal effect and are effective against lots of multidrug resistant Gram-positive pathogens[4]. The antimicrobial screening of extract and VLC fractions showed that it has good antimicrobial activity against the tested pathogenic organisms with all the fractions exhibiting good antibacterial and antifungal activities. F4 showed prominent activity against the tested organisms with MIC value of 168±0.014 µg/mL against *S. typhi* and 1886±0.032 µg/mL against *S. aureus.* F4 also demonstrated good inhibitory activity against *A. niger, C. albicans, E. coli and B. subtilis* than the other fractions or extract. F1 showed good inhibitory activity against *C. albicans* with MIC value of 197±0.014 µg/mL whereas F4 was most activity against *A. niger* (MIC = 435±0.031 µg/mL). However, fluconazole, elicited better antifungal activity than any of the fractions, with MIC values 0.380±0.034 µg/mL and 0.144±0.031 µg/mL respectively for *A. niger* and *C. albicans*. F3 showed most activity against *S. aureus* with MIC value of 417±0.022 µg/mL. All the fractions elicited good antibacterial activity against the tested bacteria pathogens with the standard drug (ciprofloxacin), having highest activity against *S. aureus* with MIC 0.594±0.026 µg/mL (Table 1).

Antioxidant Assay

The free radical scavenging activity of the extract and fractions was evaluated using the 1, 1-diphenyl-2-picryhydrazyl (DPPH) assay while the total antioxidant capacity (TAC) assay was carried out using the phosphomolybdate model. The results of these *in-vitro* models showed that the extract and VLC factions of *S. canus* ATCC 12647 have remarkable antioxidant activity. The percent antioxidant inhibitions obtained from both models were dose dependent. Our results showed that the extract produced higher antioxidant activity (64.93±0.14%) than the VLC fractions in the radical scavenging assay. This indicated that the bioactive metabolites have synergistic antioxidant potentials. Studies has shown that *S. canus* produces antibiotic like telomycin, vancomycin and resistomycin, no report of synergistic antioxidant activity of these biomolecules have been documented. But synergistic antioxidant activity of plant metabolites isolated from different plants has been reported[26]. These plant metabolites isolated from different plants belong to the polyphenolics and their antioxidant activities could be attributed to the radical scavenging potentials of polyphenolic compounds.

Compared to the ascorbic acid with percent inhibition of 72.80±0.24, all the fractions elicited remarkable antioxidant activity with F3 having the highest free radical scavenging activity (inhibition percentage = 60.66±0.04) (Table 2). Similar dose dependent antioxidant effects were also observed in the phosphomolybdate model. In this model, F1 produce higher percentage inhibition. Comparing the two models, the 1, 1-diphenyl-2-picryhydrazyl (DPPH) seems to be more sensitive than the phosphomolybdate method. This is evident in the percentage antioxidant inhibition elicited from both models. DPPH assay s more sensitive than the phosphomolybdate method since its radical scavenging activity involves donation of hydrogen atom or transfer of an electron to the nitrogen atom to scavenge the radical unlike the phosphomolybdate method[27-29].

5. Conclusion

Our results showed that extract and VLC fractions of *Streptomyces canus* ATCC 12467 demonstrated good *in-vitro* antimicrobial activity, and with remarkable antioxidant potency. Detailed and elaborate activity guided isolation, and characterization of bioactive metabolites from the most active fractions is on-going.

Acknowledgements

The authors gratefully acknowledge British Society for Antimicrobial Chemotherapy (BSAC) for funding this work through a Postdoctoral Fellowship to MO Agbo. Prof. RJM Goss, School of Chemistry, University of Saint Andrew, United Kingdom is also thanked for the provision of *Streptomyces canus* strain ATCC 12467.

Conflict of interests

We declare that there is no conflict of interest.

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يمتلك المستخلص والكسور من بكتيريا التربة (*Streptomyces canus* ATCC 12647) إمكانات مضادات الميكروبات ومضادات الأكسدة في المختبر

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ملخص

أنواع Streptomyces هي أكثر منتجي المضادات الحيوية غزارة ضمن مجموعة البكتيريا الشعاعية. تم تقييم أنشطة مضادات الميكروبات ومضادات الأكسدة في المختبر لمستخلص الميثانول (MeOH) وأجزاء الكروماتوجرافيا السائلة الفراغية (VLC) لبكتيريا التربة Streptomyces canus ATCC 12647. تم استخدام طريقة آجار لنشر البئر لفحص مضادات الميكروبات، بينما تم استخدام طرق الكسح الجذري للفوسفوموليبدات و DPPH لفحص مضادات الأكسدة. أظهر اختبار مضادات الميكروبات أنشطة ملحوظة ضد *Bacillus subtilis* و *Staphylococcus aureus* و *Candida albicans*. كما أظهر المستخلص والكسور أنشطة جيدة لمضادات الأكسدة في المختبر في كلا النموذجين. أظهرت نتائجنا أن مستخلص و VLC من العزلة البكتيرية كان لها نشاط جيد كمضاد للميكروبات ومضادات الأكسدة.

الكلمات الدالة: : *Streptomyces canus*، مضادات الميكروبات، مضادات الأكسدة، المنتجات الطبيعية، الأمراض المعدية.

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تاريخ استلام البحث 2020/12/16 وتاريخ قبوله للنشر 2022/2/14.

Jordan Journal of Pharmaceutical Sciences, Volume 15, No. 3, 2022 **DOI:** *https://doi.org/10.35516/jjps.v15i3.417*

Antibacterial and Antioxidant Potential of *Ziziphus jujube*, *Fagonia Arabica*, *Mallotus phillipensis* and *Hemidesmus Indicus*

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ABSTRACT

Phytochemicals present in plants are a major source of imparting different medicinal properties to the plant. Four medicinal plants, i.e., *Ziziphus jujube* Mill., *Fagonia arabica* L., *Mallotus phillipensis* (Lam.) Müll.-Arg. and *Hemidesmus indicus* (L.) Schult were evaluated for their antibacterial and antioxidant potentials. Chemical analysis of ethanol and ethyl acetate extract of these plants revealed the presence of various phytochemicals in them. Antibacterial activity of extracts was measured against a *Bacillus* and a *Pseudomonas* spp. Among all of the extracts, *M. phillipensis* ethyl acetate extract gave maximum zone of inhibition (14mm) against *Bacillus* spp. Minimum inhibitory concentration of *M. phillipensis* ethyl acetate extract was 62.5mg/L. *M. phillipensis* extract was found to exhibit the maximum bacterial efflux pump inhibition potential (155%). Due to these antibacterial properties, twelve components of *M. phillipensis* were separated by TLC. Out of these 12, the component showing antibacterial potential was subjected to GCMS analysis which indicated that phthalic acid was the bioactive component responsible for this activity. Antioxidant potential of all extracts was also estimated by various assays where *M. phillipensis* had maximum potential among all. In conclusion, *M. phillipensis* extract had maximum antibacterial and antioxidant potential. The bioactive components isolated from this plant can further be used in pharmaceutical industries.

Keywords: Phytochemical Analysis, Antibacterial Activity, Thin Layer Chromatography, Bacterial Efflux pump inhibition activity, Gas chromatography mass spectrometry.

1. INTRODUCTION

Plants are considered to be the most abundant creation of nature that provides protection and nutrition to almost all the living things on earth ranging from bacteria to mammals [1]. Ethnobiology is the field of science dealing with plants and animals that were used in primitive times for the benefit of mankind. It was initially described by Edward F. Castetter in University of Mexico as "utilization of plant and animal life by primitive people" [2]. In Pakistan, there are about 6000 higher plants species present due to wide-ranging climatic zones. Out of all of these plants, about 12% are used medicinally by local practitioners (Pansare) in the crude form as a drug [3].

Plants are found to have antimicrobial properties due to the bioactive components present in them. This antibacterial potential is due to the secondary metabolites produced in the plants. When plants absorb sunlight, they give off plenty of oxygen and secondary metabolites [4]. Plants usually produce secondary metabolites in response to any danger posed to them by surrounding pathogens or any other external stimuli such as environmental change or nutrition deprivation [5].

Research in ethnobotany has also promoted the study of antioxidants in these plants. These antioxidants are responsible for preventing the cells from damage caused by free radicals [6]. Many of these antioxidants are present

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Received on 16/12/2020 and Accepted for Publication on 14/2/2022.

in our body that are derived from fruits or vegetables consumed by us. Reactive oxygen species are known for causing different illnesses, for instance, cancer, diabetes, artherosclerosis, arthritis, Alzheimer disease and neurodegenerative disease [7]. Antioxidants react with these oxygen species and scavenge them to save the cells from getting deteriorated [8].

Four different plants were used in the present study for their characterization. First plant used was *Ziziphus jujube* commonly known as Unaab [9]. The Ziziphus species is utilized as a part of medication for the cure of a few disorders, for example, diabetes, diarrhea, digestive disorders, fever, insomnia, liver complaints, obesity, skin infections, urinary disorders, and weakness [10]. *Fagonia arabica* commonly known as Dhamasa Booti is a tropical herb [11]. This plant is also known to treat different common problems like boils, thirst, leucoderma, vomiting, typhoid, dysentery and asthma [12]. *Mallotus phillipensis* is generally famous as Kameela. The bark decoction of this plant is well-known for treating diseases like meningitis, diarrhea, dysentery, stomachic effect, worm and typhoid [13]. It is also reported to contain anti-allergic properties and bactericidal properties as well as against Helicobacter pylori which is a renowned chemo resistant strain [14]. Ushba, i.e., *Hemidesmus indicus*, is official medicine in Indian and British pharmacopeia. This plant is considered as a tonic, diaphoretic, blood purifier, diuretic and demulcent, i.e., relieving inflammation [15]. It is utilized in nutrition deficiency, syphilis, urinary problems, skin diseases and chronic rheumatism. It is usually used in the powder form or as water extract (decoction) [16].

The objective of this research was to identify the phytochemicals, antibacterial and antioxidative potential of four selected medicinal plant extracts that are known to be useful in treating skin infections. Phytochemicals are actually responsible for governing different properties of the plant. Identification of these phytochemicals and their potential can help the pharmaceutical companies to utilize herbs in their medicine and generate better and novel medicines for the diseases whose medication is still not available. It will help to escape the bacterial resistance issue and also aid in the production of cheaper products.

2. Results

Four plants were analyzed for their phytochemical, antibacterial and antioxidant potentials but the plant i.e. M. phillipensis that gave good potential of all of these traits was chosen to be separated into different fractions by TLC and the bioactive fraction (having antibacterial potential) was analyzed by GC-MS analysis.

2.1 Phytochemical analysis of selected medicinal plants

To check the presence or absence of different phytochemicals in extracts of *Z. jujube*, *F. arabica*, *M. phillipensis* and *H. indicus*, different biochemical tests were performed. The results of these tests are given in table 2.

2.2 Antioxidative potential of selected plant extracts

Antioxidants are the molecules that prevent the oxidation of other molecules. Antioxidant activity of any extract is regarded as the potential of its antioxidant constituents to hunt the oxidants in organism thus restraining their activity. In this study, radical scavenging ability, total phenolic content, phosphomolybdate assay, ferric reducing antioxidant potential and total flavonoid content assay were performed to analyze the ability of plant extracts. All the extracts showed some potential. *M. phillipensis* extract gave maximum potential in most of the assays such as, 69.4% Radical scavenging ability, 120GAEµg/ml total phenolic content and 7400REµg/ml total flavonoid content. While, *F. arabica* extract gave maximum ferric reducing antioxidant potential i.e. 747AAEµg/ml. phosphomolybdate assay revealed almost similar antioxidant potential of *Z. jujube* (553 AAEµg/ml), *F. arabica* (552 AAEµg/ml) and *H. indicus* (553 AAEµg/ml) extracts. The results of all of these assays are summarized in table 1.

2.3 Antibacterial activity

Antibacterial activity is termed as the capability of that

compound to constrain the growth of bacteria under its influence. Antibacterial potential of these extracts was confirmed against a gram positive *Bacillus* strain (JQ013099) and a gram negative *Pseudomonas* strain (KC881030). All the extracts showed variable inhibition of both the strains maximum inhibition zone was given by *M. phillipensis* extract extract i.e. 11mm for ethanol and 14mm for ethyl acetate extract as shown in table 3, figure 1.

Table 1: Ferric reducing antioxidant potential, phosphomolybdate assay, total flavonoid and total phenolic content of *Z. jujube, F. arabica, M. phillipensis* and *H. indicus*.

Samples		Ferric Reducing Antioxidant Potential (AAE µg/ml)	Phoshpho-molybdate Assay (AAE µg/ml)	Total Flavonoid Content (RE µg/ml)	Total Phenolic Content (GAE µg/ml)
Ethanol	**Control**	**7.16±5.13**	**44.13±0.86**	**8.62±0.28**	**8.62±0.28**
	Z. jujube	677.88±5.54	553.41±0.75	85.86±0.80	85.86±0.80
	F. arabica	747.11±4.67	552.51±0.98	80.33±0.92	80.33±0.92
	M. phillipensis	670.50±3.57	471.75±0.63	120.01±1.21	120.01±1.21
	H. indicus	676.75±3.40	553.05±0.92	46.37±0.69	46.37±0.69
Ethyl Acetate	**Control**	**6.59±2.65**	**17.44±0.64**	**2.94±0.11**	**2.94±0.11**
	Z. jujube	206.90±4.33	519.34±0.46	19.37±0.63	19.37±0.63
	F. arabica	524.67±3.40	540.25±0.30	27.24±0.46	27.24±0.46
	M. phillipensis	281.37±4.56	193.22±0.71	15.60±0.51	15.60±0.51
	H. indicus	20.49±4.73	66.12±0.93	9.33±0.17	9.33±0.17

Where, GAE= Gallic acid equivalents, AAE= Ascorbic Acid Equivalents, RE= Rutin Equivalents.

Table 2: % DPPH radical scavenging potential of *Z. jujube, F. arabica, M. phillipensis* and *H. indicus extracts at different concentrations*.

Samples		% DPPH radical scavenging potential of extracts			
		25mg/ml	**50mg/ml**	**100mg/ml**	**250mg/ml**
Ethanol	**Control**	**0.9±0.12**	**2.4±0.02**	**4.6±0.98**	**11.3±1.32**
	Z. jujube	5.4±0.09	9.5±0.97	25.1±3.27	52.6±1.44
	F. arabica	4.1±0.21	7.2±0.42	17.6±2.89	35.8±0.75
	M. phillipensis	7.2±0.52	13.5±1.81	28.3±1.74	69.4±1.78
	H. indicus	3.9±0.29	7.5±0.72	15.7±0.82	37.1±2.19
Ethyl Acetate	**Control**	**0.2±0.05**	**0.7±0.08**	**1.5±0.03**	**3.4±0.80**
	Z. jujube	1.0±0.23	2.2±0.38	5.1±0.61	12.0±2.42
	F. arabica	2.1±0.71	4.7±0.76	9.8±1.20	23.3±1.78
	M. phillipensis	5.9±0.65	13.4±0.64	25.7±2.32	61.6±2.94
	H. indicus	1.6±0.31	3.3±0.43	6.2±0.51	12.3±1.21

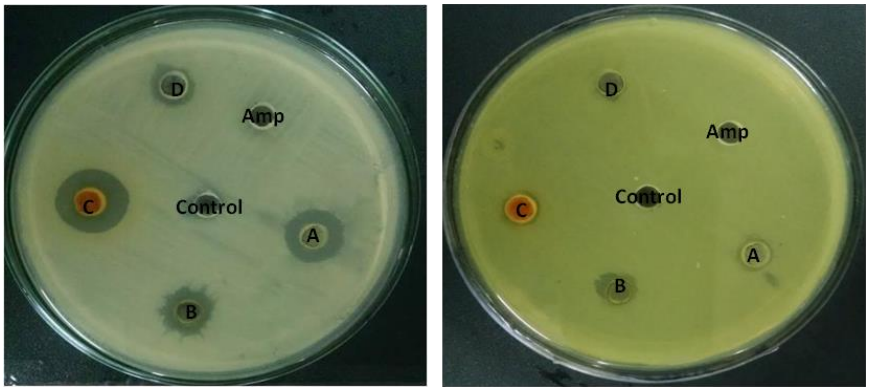

Figure 1. Antibacterial activity of ethanol extracts against gram positive (left) and gram negative (right) bacteria. A= *Z. jujube*, B= *F. arabica*, C= *M. phillipensis*, D= *H. indicus*

2.4 Bacterial efflux pump inhibition

Bacterial efflux pumps are the main helpers in imparting resistance to the bacterial cells against the antibacterial agents. Rhodamine is known to promote this activity while reserpine helps to minimize the resistance against antibacterial agents. In this study, bacterial efflux pump inhibition due to the plant extracts was observed. For this, the bacterial efflux pumps were first activated by exposing the cells to Rhodamine and then the inhibition of this resistance mechanism was studied. Reserpine was utilized as a positive control in the current study which showed 30.12% inhibition of the efflux pump. While, *M. phillipensis* ethanol extract was observed to have maximum inhibition potential of efflux pumps i.e. 155%. Results of this assay are mentioned in table 2.

2.5 Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration is the concentration of a compound which is minimally required to stop the growth of bacteria completely. *M. phillipensis* extract gave maximum inhibition potential against bacteria. For this reason, MIC of ethanol and Ethyl acetate extracts of *M. phillipensis* were determined against a gram positive and a gram negative strain. *M. phillipensis* ethanol extract resisted the growth of bacteria at minimum concentration of 1.875mg/ml. While for gram negative strain, the minimum concentration to inhibit bacterial growth was 62.5mg/ml.

2.6 Thin layer chromatography (TLC)

Thin layer chromatography is used to separate different compounds present in a mixture depending upon their solubility in the solvent system. M. phillipensis (Kameela) was found to have the maximum antibacterial potential. That is why this plant was chosen to analyze the components responsible for its antibacterial potential. Twelve spots from ethanol and seven spots from ethyl acetate extract were separated with the help of TLC.

2.6.1 Antibacterial activity of TLC spots

The compounds separated via TLC were tested for their antibacterial activity independently so that the compound responsible for the activity must be identified. The antibacterial potential of ethanol and ethyl acetate extract spots was tested against a gram-positive strain, and it was found that maximum zone sizes were given by spot 3 (R_f Value = 0.32) and 4 (R_f Value = 0.35). Spot 3 gave a diameter of about 3mm while spot 4 gave a 2mm diameter of the inhibition zone against gram positive bacterial test strain.

2.7 GCMS Analysis

GCMS is a chromatographic technique used to identify, separate or quantify the compounds present in a mixture. The purpose of GCMS analysis in this study was to identify bioactive compounds that possessed antibacterial activity in Mallotus phillipensis ethyl acetate extract, on the basis of their mass. Analysis of these

compounds showed that spots 3 and 4 both had their maximum peaks at about 31 minutes retention time. Mass analysis of these compounds revealed them to be phthalic acid in both of the spots as shown in figure 2.

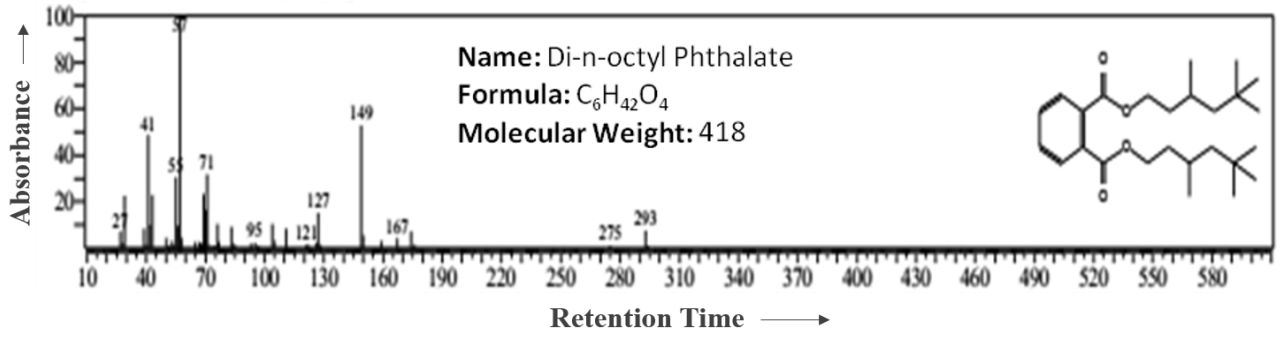

Figure 2: GCMS analysis showing the structure of phthalate isolated from Mallotus philippensis extract.

Table 3: Phytochemical analysis of Z. jujube, F. arabica, M. phillipensis and H. indicus.

Extracts		Phytochemical Tests										
		Alkaloids	Carbohydrates	Cardiac Glycosides	Flavonoids	Phenols	Phlobatannins	Reducing sugars	Saponins	Steroids	Tannins	Terpenoids
Ethanol	N	-	-	-	-	-	-	-	-	-	-	-
	A	-	+	+	+	+	-	+	+	+	+	+
	B	+	+	-	+	-	+	+	+	+	-	+
	C	+	+	+	-	+	+	+	+	+	+	+
	D	-	+	+	+	-	-	+	+	+	-	+
Ethyl Acetate	N	-	-	-	-	-	-	-	-	-	-	-
	A	-	+	-	-	-	-	-	-	-	-	-
	B	+	+	+	+	-	+	-	-	+	-	+
	C	+	+	+	-	-	+	+	+	+	-	+
	D	-	+	-	+	-	-	-	+	+	-	+

N= Control, A= *Z. jujube*, B= *F. arabica*, C= *M. phillipensis*, D= *H. indicus*

Table 4: Antibacterial and efflux pump inhibition potential of *Z. jujube, F. arabica, M. phillipensis* and *H. indicus*.

Extracts		Antibacterial Activity*		Bacterial efflux pump inhibition% **
		Gram Positive (mm)	Gram Negative (mm)	
Ethanol	**N**	**01±0.08**	**00±0.00**	**18.00±1.15**
	A	09±0.12	2.5±0.22	43.21±1.73
	B	07±0.44	02±0.25	75.69±0.57
	C	11±0.59	03±0.12	155.66±2.30
	D	04±0.56	01±0.22	39.40±1.73
Ethyl Acetate	**N**	**08±0.51**	**01±0.07**	**5.86±0.57**
	A	02±0.35	05±0.03	15.5±2.30
	B	12±0.03	01±0.53	4.06±1.15
	C	14±0.17	02±0.35	5.15±1.73
	D	05±0.28	02±0.48	2.50±2.88

N= Control, A= *Z. jujube*, B= *F. arabica*, C= *M. phillipensis*, D= *H. indicus*

Mean of replicates, ± Standard error of mean

*Ampicillin (10µg/ml) was used as a standard and all of the extracts were resistant to that antibiotic concentration.

**Reserpine was used as a standard and the %inhibition observed from it was 30.12±1.

Discussion

Plants are known to be utilized for medicinal purposes for thousands of years [17]. In this study, ethanol and ethyl acetate extracts of some medicinal plants were analyzed for their antibacterial and antioxidant properties. Upon phytochemical detection, ethanol extracts were found to have more phytochemicals in them as compared to the ethyl acetate extracts. This difference in the composition of extracts can be attributed to the fact that different compounds dissolve in different solvents depending upon the polarity of solvent [18]. Ethyl acetate is a solvent that mostly just leaches in to the skin of the plant and removes the chemicals that are present superficially while ethanol is known to rupture the cell membranes and thus extracts the intracellular components of the plants [19].

Carbohydrates, terpenoids and steroids were found in almost each of the plant extracts in both the solvents. Reducing sugars and saponins were present in all of the plant extracts of ethanol while other phytochemicals such as alkaloids, cardiac glycosides, flavonoids, phenols, phlobatanins and tannins were not found in all of the extracts. This finding was not in accordance to the finding of Fazali and co-researchers, because according to their study alkaloids, flavonoids, terpenoids, phlobatanins, and tannins are present in most of the plants but in our tests these phytochemicals were not found in all of the extracts whereas carbohydrates, terpenoids and steroids were present in almost all of the extracts [20].

According to various studies on phytochemicals, it is reported that they are known to protect the body from diseases that are induced by oxidative stress. This oxidative stress causes the release of different free radicals and other reactive oxygen species in the body, thus causing various harmful diseases. In that case, antioxidants in plants help to scavenge these harmful oxygen species thus reducing the negative effect of oxidative stress related diseases [21].

DPPH free radical scavenging assay was performed on the extracts of selected plants. Ethanol extracts were found to have more antioxidants in them as compared to the ethyl acetate extracts. In both of the solvents *M. phillipensis* extract was having maximum potential. This can be due to

the fact that *M. phillipensis* ethanol extract contained maximum phenolic content in comparison with other extracts. As, according to a research, phenolic compounds act as a free radical terminator thus enhancing the radical scavenging ability of the plant [22].

Extracts in both solvents i.e. ethyl acetate and ethanol were tested for their phenolic content. Ethanol extracts had higher content than ethyl acetate. This relation can be attributed to the fact that ethanol solvent has greater potential to dissolve antioxidants in it [23]. *M. phillipensis* extract showed maximum phenolic content and this observation is in correspondence with the study of Ziaul Haque who has reported the presence of different phenols in *M. phillipensis* plant i.e. mallotophilippinens, bergenin, isorottlerin and rottlerin [24].

Phosphomolybdate assay was done to estimate the total antioxidant capacity of extracts. In ethanol, all the extracts showed almost similar concentration except *M. phillipensis* extract. While in the case of ethyl acetate extracts *Z. jujube* and *F. arabica* showed almost same concentration while *H. indicus* had minimum concentration and *M. phillipensis* had concentration in between these three extracts. According to a previous research there is an inverse relationship between total phenolic content and the antioxidative potential of plant extracts estimated through phosphomolybdenum assay [25]. This previous research is in compliance with current study as, *M. phillipensis* extract showed maximum phenolic content but in phosphomolybdate assay its antioxidative potential was lesser than other extracts.

Ferric reducing antioxidant potential assay was also performed on extracts, just like other antioxidant tests, ethanol extracts showed more potential of ferrous reduction. According to a research conducted by Dudonné et al, there is a noteworthy association among FRAP, total phenolic and DPPH radical scavenging properties. Contrary to Dudonné's research, this relation was not found to be linear in this study [26].

Total flavonoids content of the plants was calculated by using Aluminum chloride method. When total flavonoids concentration of the extracts was determined, the ethanol extracts showed lesser concentration than the ethyl acetate extracts this observation was similar to the study of Ramammoorthy and Bono who observed greater flavonoid content in ethyl acetate extracts as compared to ethanol extracts [27].

Antibacterial potential of chosen medicinal plants was tested against a gram positive and a gram-negative bacterial strain. All of the extracts showed greater inhibition potential for gram positive strain than gram negative strain. This difference in the antibacterial activity can be due to the fact that gram negative bacteria possess an extra membrane that surrounds the cell wall. This membrane restricts the entry of extracellular compounds from the lipopolysaccharide covering of the cell, thus limiting the potential of chemicals to inhibit bacterial growth. Other than outer membrane, there are some enzymes as well, present in periplasmic membrane that help in breaking down the foreign molecules coming from outside of the cell as a result of which bactericidal effect of chemicals is constrained [28].

Apparently, ethyl acetate extracts showed greater inhibition potential than ethanol extracts. But the zone of inhibition of ethyl acetate control well was 8mm which means if the value of control zone of inhibition is deducted from extract's zone of inhibition, the antibacterial activity due to plant constituent itself would be very low. While in the case of ethanol control well, the zone of inhibition was not much significant which depicted that maximum of the inhibition observed by ethanol extracts was due to the bioactive compounds in them rather than the solvent. As reported by Lou and co-researchers, lesser antibacterial potential of ethyl acetate extracts can be attributed to lower total phenolic content of the ethyl acetate fractions [29]. While, high inhibition capacity of ethanol extracts can be reported due to the better dissolving potential of ethanol [30]. This inhibition of bacterial growth can be due to the presence of flavonoids in plant extracts. Because,

according to a study on antibacterial properties of flavonoids it was reported that flavonoids are a good inhibitor of bacteria. They inhibit the bacterial cell growth by acting on their membranes [31].

Utilizing well diffusion method for antibacterial potential estimation is not always appreciated because this method may show varied results depending upon the penetration power of the constituents. To combat this problem, broth micro-dilution method is a good choice. It gives more detailed results i.e. the quantitative analysis [28]. Tetrazolium salt was used in this study to enhance the bacterial growth sensitivity. As soon as this salt comes in contact with the terminal electron of electron transport chain, it produces color. This terminal electron is liberated only from the electron transport chain being processed by the live cells in the broth. It could be observed in this study that the wells in which concentration of plant constituents was sufficient to inhibit the bacterial growth did not show any change in color. While, the color of broth changed to pink in the wells where phytochemicals were so diluted that they could not produce bactericidal effect [32].

Antibacterial potential of the extracts was also estimated by targeting the efflux pump mechanism of bacteria. Rhodamine dye was used to measure the efflux pump potential of bacteria. In the presence of any efflux pump inhibitor, such as plant extract, the activity of bacterial efflux pumps should be lessened. For this reason, reserpine was used as a standard. Reserpine reduced the efflux of rhodamine dye by 30%. The potential of ethyl acetate extracts was even lesser than the reserpine activity which showed that it was not much active in deactivating the efflux pumps of bacteria. In compliance to this study some researchers also suggest that this activity can be due to lesser potential of ethyl acetate to dissolve phytoconstituents responsible for efflux pump inhibition. While, ethanol being a good solvent gave high activity [33].

Thin layer chromatography of *M. phillipensis* extracts of ethanol and ethyl acetate was performed to separate the different components present in these extracts. When these TLC plates were developed in respective solvent systems, both of the plates gave a brown band upon treatment with H_2SO_4. In compliance with a research by Chavan and Amarowicz, dark brown bands demonstrate the presence of sugars in these extracts [34]. While another study demonstrates the presence of different amino acids, if yellowish brown to brown colored bands appear after treatment with ninhydrin spray. These yellowish brown colored bands were observed in both plates indicating the presence of different amino acids [35].

GCMS analysis was carried out to identify the bioactive compounds in *Mallotus phillipensis* that were responsible for imparting antibacterial activity to this plant. The antibacterial activity observed by these spots was lesser than the activity observed when whole plant was subjected to the test. This could be attributed to lesser concentration of the plant components because upon separation by TLC some of the fraction might be lost during scratching or extraction. It could also be due to the synergistic effect of whole plant components as, more than one plant extract's fraction was observed to produce antibacterial effect. Basic component that was identified via GCMS analysis is phthalic acid in both of the spots. Presence of this component in *M. phillipensis* plant has also been reported by other researchers [36].

It can be concluded that, the medicinal plants *Z. jujube*, *F. arabica*, *M. phillipensis*, and *H. indicus* used in this study were found to have antibacterial and antioxidant potential due to the presence of certain phytochemicals in them. *M. phillipensis* was observed to have maximum potential among all extracts. These potential phytochemicals can prove to be pharmacologically beneficial because these phytochemicals can be isolated from crude extracts for manufacturing new synthetic drugs. Future studies should be focused on the detailed analysis of phytochemcial constituents of these medicinal plants. Phytochemicals responsible for their medicinal properties should be isolated and modified for pharmacological purpose.

3. Methods

3.1 Collection of medicinal plants

Plants known for treating skin infections were purchased from a local store in Lahore, Pakistan. These plants included Unaab (*Ziziphus jujube* Mill.), Dhamasa Booti (*Fagonia arabica* L.), Kameela (*Mallotus phillipensis* (Lam.) Müll.-Arg.) and Ushba (*Hemidesmus indicus* L.). These plants were identified by Professor Dr. Sikandar Sultan (Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore). These plants were analyzed and stored in Institute of microbiology and molecular genetics (University of the Punjab) under the voucher numbers MMG-IM-20, MMG-IM-21, MMG-IM-22 and MMG-IM-23. Whole plant of *F. arabica* and *H. indicus* were used for this study. While, *Z. jujube* flowers and *M. phillipensis* seeds were utilized for their analysis.

3.2 Preparation of Plant Extracts

Selected medicinal plants were washed under distilled water, dried and ground into powder form. For the extraction plant to solvent ratio was kept at 1:4. These flasks were left in dark overnight. Next day, these soaked plants were filtered with Whatmann filter paper no. 1 to obtain the extract containing phytochemicals. These extracts were evaporated using Hei-vap-series, Heidolph Germany rotary evaporator. The powdery material obtained after evaporation was stored in dried form and was further diluted as per need.

3.3 Phytochemical screening of selected medicinal plants

The selected medicinal plants were checked for the presence of different phytochemical components in them. The selected medicinal plants were checked for the presence of different phytochemical components in them. Estimation of alkaloids, carbohydrates, cardiac glycosides, flavonoids, phenols, phlobatanins, reducing sugars, saponins, steroids, tannins and terpenoids [37-39].

Alkaloids

A mixture of 1ml extract and 1ml 1% HCL was boiled in a water bath. After that, 1ml of Wagner's reagent was added to the mixture which gave red precipitates indicating positive results.

Carbohydrates

In a test tube, 1ml of extract was mixed with 1ml Molisch's Reagent and 1ml conc. sulphuric acid. The mixture was left to stay for few minutes and a red or violet ring at the interface of two layers was observed as a positive result.

Cardiac glycosides

Half ml of glacial acetic acid with a drop of ferric chloride was added to 1 ml of extract. To this mixture 0.5ml of conc. sulphuric acid was added. A brown ring development indicated positive result for cardiac glycoside in extracts.

Flavonoids

For the estimation of flavonoids in the extract, one ml of extract was mixed with 0.5ml of 20% sodium hydroxide. The mixture turned yellow. These results were cross checked by the addition of dilute HCl which caused the color to fade away.

Phenols

Appearance of deep blue or black color when 0.5ml of 5% ferric chloride solution was mixed with 1ml of extract indicated the presence of phenols.

Phlobatannins

In a boiling water bath, a test tube containing 1ml extract and 1ml 1% HCl, was placed. The reaction resulted in production of red precipitates which indicated the presence of phlobatannins.

Reducing sugars

One milliliter extract was dissolved in 1ml of distilled water in a tube. In another tube, 1ml of Fehling Solution A and B each were added and boiled in water bath. This solution was poured in extract and color change was observed as a positive test indication.

Saponins

Saponins presence in extracts was indicated by the stable foam formation when 1ml of extract and 1ml of distilled water were mixed and shaken vigorously.

Steroids

One ml of chloroform was mixed with 1ml of extract and 1ml of conc. H_2SO_4. Production of red color in the lower layer of chloroform indicated the presence of steroids.

Tannins

One milliliter of extract was added in 1ml of distilled water followed by a few drops of ferric chloride solution. Green colored precipitates indicated the existence of tannins in extracts.

Terpenoids

One milliliter of extract was mixed in one ml of chloroform and was left to evaporate. One ml of H_2SO_4 (concentrated) was then added and the mixture was heated for 2 minutes. Formation of grey color indicated the presence of terpenoids in extracts.

3.4 Antioxidative potential of plant extracts

Oxygen species produce oxidative stress in the body because number of oxidants increase as compared to antioxidants thus causing harm to biomolecules like proteins, lipids and DNA [24]. Antioxidative properties of plant extracts can be determined by different assays including, DPPH free radical scavenging ability, total ohenolic content, phosphomolybdate assay, ferric reducing antioxidant potential and total flavonoids content [40]. In the selected plants, presence of antioxidants was detected by following different assay protocols reported by [41].

3.4.1 DPPH free radical scavenging ability

For this assay, a stock solution of DPPH free radical was prepared by adding 24mg of DPPH in 100ml of methanol. From this stock, working solution was prepared by diluting it with methanol until the absorbance of solution became 0.98±0.02 at 517nm. In a test tube, 3ml of this working solution was added along with 100µl of extract and mixed well. These test tubes were placed in dark for 30 minutes. After incubation, the test tubes were taken out and absorbance of solution was taken at 517nm. Percent activity of these extracts was estimated using following formula:

% Radical scavenging ability =

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

3.4.2 Total phenolic content

In a test tube 300µl sample was taken and 1ml of respective solvent was further added in it. In that tube, 3.16ml of distilled water was added along with 200µl of Folin Ciocaltaeu reagent and incubated for 8 minutes. After incubation, 600µl of 10% sodium bicarbonate was added and tubes were covered with aluminum foil. Covered tubes were placed in water bath for 30 minutes preset at 40° C. After that, absorbance was taken at 765nm. Same procedure was done for gallic acid standard curve formation for calculating gallic acid equivalent concentration of the phenols in extracts.

3.4.3 Phosphomolybdate assay for total antioxidant capacity

Phosphomolybdate reagent was prepared by mixing 28mM sodium phosphate, 4mM ammonium molybdate and 0.6M sulfuric acid. Then 3ml of this reagent was added to 300µl of plant extract and the tubes were covered with aluminum foil. These tubes were incubated for 90 minutes at 95˚C. After incubation, absorbance was taken at 765nm. In this method ascorbic acid standard was used to make a standard curve from the concentration of 25mg to 500mg/L. The total antioxidant potential was assessed by this formula: [42]

Antioxidant capacity (%) =

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

3.4.4 Ferric reducing antioxidant potential

For this assay, 25ml of 30mM Acetate buffer, 2.5ml of 10mM TPTZ solution and 2.5ml of 20mM Ferric chloride solution were mixed together to make FRAP reagent. Then, 2.85ml of this FRAP reagent was added in a tube containing 150µl of extract. Tubes were put at dark place for 30 minutes and finally optical density was taken at 593nm. Ascorbic acid

was used as a standard and the same process was done for ascorbic acid (50mg- 500mg/L) to make a standard curve. Then the ascorbic acid equivalent (AAE) concentration of extracts was estimated using standard curve.

3.4.5 Total flavonoid content

Three hundred microliter of extract and 3.4ml of 30% solvent was mixed in a test tube and 150µl of 0.5M sodium nitrite and 0.3M aluminum chloride each were added in it. The tubes were left at room temperature for 5 minutes. Then, 1ml of 1M sodium hydroxide solution was added and mixed well. Absorbance of these tubes was taken at 506nm. Rutin was used as standard in this test and standard curve was made by using the similar procedure and taking the amount of rutin from 75 to 750mg/L.

3.5 Estimation of antibacterial activity of extracts by agar well diffusion method

For this purpose, nutrient broth and Mueller Hinton agar was used. The procedure of bacterial growth and agar well diffusion reported by Dilshad et al was used [43]. Ampicillin (10µg/ml) was sued as a standard antibiotic. Ethanol and ethyl acetate were used as negative controls.

3.6 Bacterial efflux pump inhibition (EPI)

Efflux pumps are actually the transport proteins which help in transporting toxic substances out of the cell into the extracellular environment [44]. In this study, plant extracts were studied for testing their EPI potential. For this purpose, the method of Sewanu et al was used with minor modifications [45]. Optical density of final supernatant was taken at 527nm and the activity (in percentage) of these extracts was estimated by using following formula.

$$\textbf{Percent efflux pump inhibition activity} = \frac{1-At}{Ao} \textbf{x100}$$

Where, At = Absorbance of test

A_o = Absorbance of Control

3.7 Minimum inhibitory concentration

Minimum inhibitory concentration estimation is a quantitative assay for determining the minimum extract concentration that will be able to restrain the bacterial growth [46]. This assay was done by using the method of Klancnik *et al* with some modifications [28]. Penicillin (2µg/ml), erythromycin (2µg/ml), tetracycline (2µg/ml) and chloramphenicol (2µg/ml) were used as standards in this protocol.

3.8 Thin layer chromatography

Thin layer chromatography is a method used for separating compounds from extract of any plant [46]. It helps in compound identity, purity and quantification [47]. *M. phillipensis* extract of both solvents were subjected to TLC for identifying the components present in whole extract. Method of Dilshad et al was used here with slight modifications [43]. For ethanol extract, only chloroform was used as a solvent system. While for ethyl acetate extract, chloroform and ethyl acetate were used in combination i.e. 5:1. These developed plates were then observed under high (366nm) and low UV (254nm). The plates were developed in triplicate and treated differently. One plate was treated with sulfuric acid 10% and put in oven for 10 minutes for observing the presence of sugars in extracts. Second plate was treated with ninhydrin solution and heated for observing protein compounds. Third plate was treated with iodine to observe the presence of iodine active compounds.

3.9 Antibacterial Activity of Spots

These dried separated components of plants were then subjected to antibacterial activity analysis. For this purpose, disc diffusion assay was conducted. Filter paper discs were autoclaved and put in the tubes containing extract components that were dissolved in a few micro liters of solvent. Mueller Hinton agar plates were spread with bacterial strain. Discs were taken out of tubes and air dried. Dried discs were placed on respective plates and the plates were incubated at 37^0C for 24 hours. After incubation period, the plates were analyzed for inhibition zone diameter against bacterial strain. Inhibition zone against bacteria predicted the component responsible for antibacterial activity of that extract.

3.10 Gas Chromatography Mass Spectrometry (GCMS)

Gas chromatography mass spectrometry analysis of the spots that had maximum antibacterial activity was done. The protocol of Snageetha et al was followed here [48].

Acknowledgement

Authors are thankful to University of the Punjab, Lahore for providing financial assistance for the completion of this research work. This research paper is a part of MS thesis of Ms. Rimsha Dilshad.

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مضاد للجراثيم ومضادات الأكسدة المحتملة من زيزيفوس عناب، فاغونيا أرابيكا، مالوتوس فيليبينسيس وهيميديسموس إنديكوس

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ملخص

المواد الكيميائية النباتية الموجودة في النباتات هي مصدر رئيسي لإضفاء خصائص طبية مختلفة على النبات. أربعة نباتات طبية، أي زيزيفوس عناب مطحنة، فاغونيا أرابيكال، مالوتوس فيليبينسيس (لام.) إم إرمل.– ارج. وهيميديسموس إنديكوس (ل.) تم تقييم شولت لإمكانياتها المضادة للبكتيريا ومضادات الأكسدة. كشف التحليل الكيميائي للإيثانول ومستخلص أسيتات الإيثيل لهذه النباتات عن وجود العديد من المواد الكيميائية النباتية فيها. تم قياس النشاط المضاد للبكتيريا من مقتطفات ضد عصية والزائفة النيابة. من بين جميع المقتطفات، م. فيليبينسيس إيثيل خلات استخراج أعطى أقصى منطقة تثبيط (14 ملليمتر) ضد عصية سب. الحد الأدنى للتركيز المثبط لمستخلص أسيتات الإيثيل فيليبينسيس كان 62.5 ملغم / لتر .تم العثور على مستخلص فيليبينسيس لإظهار أقصى قدر من تثبيط مضخة التدفق البكتيري (155٪). بسبب هذه الخصائص المضادة للبكتيريا، تم فصل اثني عشر مكونات م.فيليبينسيس من قبل تلك. من بين هذه 12، تعرض المكون الذي يظهر إمكانات مضادة للبكتيريا لتحليل غمس الذي أشار إلى أن حمض الفثاليك كان المكون النشط بيولوجيا المسؤول عن هذا النشاط. كما تم تقدير إمكانات مضادات الأكسدة لجميع المستخلصات من خلال فحوصات مختلفة حيث كان لدى م. فيليبينسيس أقصى إمكانات بين الجميع. في الختام، لمستخلص م. فيليبينسيس أقصى إمكانات مضادة للبكتيريا ومضادات الأكسدة ويمكن استخدام المكونات النشطة بيولوجيا المعزولة من هذا النبات في الصناعات الدوائية.

الكلمات الدالة: التحليل الكيميائي النباتي، النشاط المضاد للبكتيريا، كروماتوغرافيا الطبقة الرقيقة، نشاط تثبيط مضخة التدفق البكتيري، قياس الطيف الكتلي اللوني للغاز.

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تاريخ استلام البحث 2020/12/16 وتاريخ قبوله للنشر 2022/2/14.

Evaluation of *Basella alba* L. Mucilage as a Suspending Agent in Metronidazole Suspension

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ABSTRACT

In the quest for natural biodegradable, non-toxic polymers for use as excipients in pharmaceutical formulations, mucilage of *Basella alba L* (BAM) stem was isolated and evaluated as a suspending agent in metronidazole suspensions at different concentrations (0.5% - 2% w/v) in comparison to tragacanth (TCG) and gelatin gums (GLT). The micromeritic properties of the mucilage powder were determined and the metronidazole suspension was characterized using flow rate, redispersion number, sedimentation volume, viscosity and pH. The degree of flocculation was also determined. BAM powder has good flow property with minimal swelling. The order of flow rate of metronidazole suspension was BAM=TCG>GLT while sedimentation volume ranking was TCG>BAM>GLT. There was no significant difference ($p>0.05$) in the redispersion number of BAM and TCG formulations. The viscosities of formulations containing BAM and TCG at concentrations of 0.5%-1.0% w/v were the same. The pH of the suspensions ranged from 5 to 8. The degree of flocculation was in the order GLT>BAM>TCG. From our findings, BAM can be used as an alternative suspending agent in suspension formulation.

Keywords: *Basella alba*, Suspensions, Suspending agents, Metronidazole.

INTRODUCTION

A Pharmaceutical suspension is a two-phase system with uniform dispersion of finely divided solid drug particles in a continuous phase of solid, liquid or gas in which the drug has minimal solubility. The insoluble solid remain in equilibrium with a saturated solution of the solid in the continuous phase. Drugs are formulated as suspension to enhance the stability of drugs that are unstable in aqueous medium and also to provide a means of formulating poorly soluble and indiffisuble drugs in liquid preparations. This system is thermodynamically unstable; hence, there is need for a stabilizer in form of a suspending agent to reduce the settling rate of the suspended particles [1]. Suspending agents can be classified into three groups namely synthetic e.g. carbopol, semisynthetic e.g. methyl cellulose and natural agents such as the polysaccharides[2-4].

Basella alba L. (Family Basellaceae) is a widely cultivated cool season vegetable and its native to tropical Southern Asia[5]. It is highly abundant in tropical Africa, Malaysia, the Caribbean, Philippines and tropical South America[6]. *Basella alba* is also known as Indian spinach, Ceylon spinach, vine spinach and Chinese spinach[7,8]. It is widely grown in the coastal area of southern Nigeria as a vegetable for food[9]. Extracts from this plant has been shown to have wound healing activity, anti-inflammatory and antiviral properties[10-12]. Mucilage from Basella leaves has been isolated and used as a suspending agent[13], a

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Received: 5/1/2021 Accepted:14/2/2022.
DOI: *https://doi.org/10.35516/jjps.v15i3.418*

binder in paracetamol formulation[14] and a disintegrant[15]. Literature search showed that mucilage from the stem was characterised[16], but there was little or no formulation studies. Therefore, this present work isolated the mucilage from the stem of *Basella alba* and its suspending properties evaluated in metronidazole suspension.

Materials and Methods

Materials

Metronidazole was a gift from Bond Chemicals, Gelatin (Type B) and tragacanth (MW 840 kDa) were obtained from BDH chemicals, UK. Indian spinach stems were obtained from a local farm in Osun State, Nigeria and authenticated at the herbarium of the Pharmacognosy Department, Olabisi Onabanjo University. All other materials were analytical grade.

Extraction of mucilage

The stems of *Basella alba* L were collected and sun dried for 10 days. The dried stems were pulverised and defatted using petroleum ether (60-80ºC). The defatted powder was soaked in distilled water for 6 hours and refluxed on water bath at 70ºC for 2 hours. It was filtered with a muslin cloth and the filtrate was precipitated with acetone and dried in hot air oven at 40ºC. Dried mucilage was pulverised and passed through sieve size No 60 (250 µm).

Characterisation of Basella mucilage

The powdered mucilage was visually examined for colour, odour and texture.

Percentage yield

The percentage yield was calculated from the equation:

$$\%\ Yield = \frac{weight\ of\ dried\ mucilage}{weight\ of\ dried\ stem} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

Hydration capacity

One gram of powdered mucilage was weighed into a 10 mL test tube, tapped and the volume (V_1) noted. Distilled water was added and made up to 10 mL mark. It was shaken for 2 minutes and allowed to stand for another 10 minutes. Volume occupied by the sediment was noted (V_2). The tube was centrifuged at 3000 rpm for 10 minutes and the supernatant was decanted. The weight of the sediment was used in calculating the hydration capacity using equation 2[17,18]: Determinations was done in triplicate

$$Hydration\ Capacity = \frac{(weight\ of\ tube + sediment) - weight\ of\ tube}{Sample\ weight\ (dry\ basis)} \quad \ldots\ldots\ldots\ldots 2$$

Determination of bulk and tapped densities

Five grams of powdered mucilage was weighed into a 50 mL measuring cylinder. The volume (bulk) occupied was noted. The measuring cylinder was subjected to 100 times of tapping manually and the tapped volume was noted. Measurements were done in triplicate. Hausner's ratio was determined from the values of bulk and tapped densities [19].

$$Bulk\ Density\ (g/mL) = \frac{Weight\ of\ mucilage\ (g)}{Bulk\ volume\ (mL)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3$$

$$Tapped\ Density\ (g/mL) = \frac{Weight\ of\ mucilage\ (g)}{Tapped\ volume\ (mL)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 4$$

$$Hausner's\ Ratio = \frac{Tapped\ Density}{Bulk\ Density} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 5$$

Angle of repose

Five gram of *Basella* mucilage was poured into a glass funnel held in place. The powder was allowed to flow through the funnel orifice (clamped with its tip 2 cm above a paper placed on a flat horizontal surface) to form a conical heap. The height of the cone and radius were determined. Angle of repose was calculated from the equation:

$$Tan\ \emptyset = \frac{height}{radius} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 6$$

All determinations was in triplicate

Determination of flow rate of suspending agents

Five gram of sample was poured into a glass funnel held in place. The powder was allowed to flow through the funnel orifice and the time taken for the flow was determined. This was done in triplicate.

Preparation of Metronidazole suspensions

Two hundred (200) mL batch sizes of 40 mg/mL metronidazole (10 µm) suspensions were prepared by dispersions method using different concentrations (0.5%-2% w/v) of the powdered mucilage as suspending agent. 0.01% w/v benzoic acid was used as a preservative. Gelatin and tragacanth gum were also used as suspending agents to compare with *Basella alba m*ucilage (Table 1).

Table 1: Formula for Preparation of Metronidazole Suspensions

Formulation	Metronidazole (g)	Basella (g)	Tragacanth (g)	Gelatin (g)	Benzoic acid (g)	Water (mL)
F1	4	0.5	-	-	1	100
F2	4	1.0	-	-	1	100
F3	4	1.5	-	-	1	100
F4	4	2.0	-	-	1	100
F5	4	-	0.5	-	1	100
F6	4	-	1.0	-	1	100
F7	4	-	1.5	-	1	100
F8	4	-	2.0	-	1	100
F9	4	-	-	0.5	1	100
F10	4	-	-	1.0	1	100
F11	4	-	-	1.5	1	100
F12	4	--	-	2.0	1	100

Determination of pH

The pH of each suspension was determined with a pH meter after preparation

Determination of sedimentation volume

50 mL of metronidazole suspension was poured into a measuring cylinder and left standing undisturbed at room temperature. At predetermined time intervals, the sedimentation volume was determined. The sedimentation volume (F) was calculated from the equation:

$$F\ (\%) = \frac{V_U}{V_O} \times 100 \quad \text{.....7}$$

V_U is ultimate volume of sediment, V_O is the original volume of suspension

Determination was in triplicate

Determination of flow rate and viscosity of suspensions

The time taken for 5 mL suspension to flow through a 5 mL pipette was determined and used in calculating the flow rate.

$$Flow\ rate\ (mL/\text{sec}) = \frac{Volume\ of\ suspension\ (mL)}{Flow\ time\ (Seconds)} \quad \text{.....8}$$

The viscosity of the suspension was determined with a Brookfield viscometer Model DV-II+Pro (Brookfield Engineering Laboratories, INC, Middleboro, MA, USA), at 25ºC using spindle 3 at 50 rpm. Determinations were made in triplicate.

Table 2: Physicochemical Properties of Suspending Agents

Properties	Basella	Tragacanth	Gelatin
Swelling index	0.85±0.04	1.80±0.56	None
Hydration capacity	3.70±0.15	4.2±0.28	None
Bulk density(g/mL)	0.357±0.01	0.385±0.02	0.385±0.01
Tapped density(g/mL)	0.385±0.01	0.455±0.02	0.500±0.01
Hausner's ratio	1.08	1.19	1.30
Angle of repose	24.90	19.65	19.65
Flow rate(g/sec)	0.1	0.2	0.2

Data are presented as the mean ± SD; n=3

Redispersion Number

The metronidazole suspensions were left to stand for one week, after which the bottles were inverted manually to allow the suspensions to re-disperse. The number of times the containers were inverted before the bottom of the bottles was free of sediments was recorded as the redispersion number.

Degree of flocculation

The method of Kumar *et al.*[20] was used for the determination of degree of flocculation. Potassium dihydrogen phosphate (0.004 mol) was added to the suspension as a flocculating agent. The sedimentation volume of the flocculated suspensions was compared with those without a deflocculating agent.

$$\beta = \frac{Sedimentation\ volume\ of\ flocculated\ suspension}{Sedimentation\ volume\ of\ deflocculated\ suspension} \quad \ldots\ldots\ldots\ldots 9$$

Statistical analysis

Statistical analysis was carried out using analysis of variance with computer software GraphPad Prism® 4 (GraphPad Software Inc. San Diego, USA).

Results and discussion

Micromeritic properties of suspending agents

The *Basella* mucilage powder was dark brown in color with a coffee-like odour. The yield was 4% w/w. The physico-chemical properties of the different suspending agents are presented in Table 2. Bulk density is the ratio of weight of powder to volume occupied which includes the inter-particulate space. The ranking of bulk density was TCG = GLT > BAM. Hausner's ratio and angle of repose are indirect ways of measuring powder flow. The ranking of Hausner ratio was BAM < TCG < GLT. Hausner ratio of less than 1.20 is indicative of good flow[21]. This indicates that BAM has good flow properties with Hausner ratio of 0.93 (Table 2). The swelling index and hydration capacity of BAM was lower than that of TCG, while gelatin did not hydrate nor swell. The ranking of flow rate of the suspending agents was BAM<TCG=GLT

Table 3: Flow rate and viscosity of suspensions

Suspending agent	Concentration (%w/v)	Flow rate ($mlsec^{-1}$)	Viscosity(centipoise)
BAM	0.5	1.11±0.19	600
	1.0	1.11±0.27	600
	1.5	0.83±0.06	600
	2.0	0.83±0.08	600
TCG	0.5	1.11±0.17	600
	1.0	1.11±0.19	600
	1.5	1.00±0.09	700
	2.0	1.00±0.09	800
GLT	0.5	1.00±0.10	400
	1.0	1.00±0.08	600
	1.5	1.00±0.09	600
	2.0	1.00±0.07	700

Evaluation of suspension

The pH of the metronidazole suspensions produced with the different suspending agents was in the range of 5 and 8. There was no change in pH with change in concentration of suspending agents. The ranking of the pH was BAM>TCG>GLT. The pH of BAM was 8.0, indicating alkaline pH. This suggests that the gum is basic. World Health Organisation recommended a pH of 5-6.5 for metronidazole suspension[22].

The flow rate of the suspensions was observed to decrease with increase in concentration of suspending agents. Similar trend was observed by Bamiro *et al.* [23] when terminalia gum was used as a suspending agent in magnesium carbonate formulation. Oppong *et al.*[24] also observed the same trend with shea tree gum in paracetamol suspension formulation. The ranking of flow rate at 0.5% and 1.0% w/v concentration was BAM=TCG>GLT. This indicates that BAM suspensions have better flow ability than GLT suspensions. A good suspension must be easily pourable from a container, therefore from this result; we can infer that BAM has the quality of a good suspending agent.

The suspensions were observed to sediment rapidly during the first 24 hours, after which the sedimentation became steady. This can be seen in the representative plot shown in figure 1. The ranking of sedimentation volume was TCG>BAM>GLT. A suspension with sedimentation volume ratio of 1 or 100% is said to be a good suspension[25]. High sedimentation volume is an indication that even though the particles have settled, as expected with suspensions, the interparticle attraction and bonding were loose and not strong enough to form hard cake during the study period[26]. Suspensions containing GLT had the least sedimentation volume. A good suspension is expected not to sediment rapidly. When sedimentation volume is near to 1 or 100%, the particles tend to flocculate easily[4].

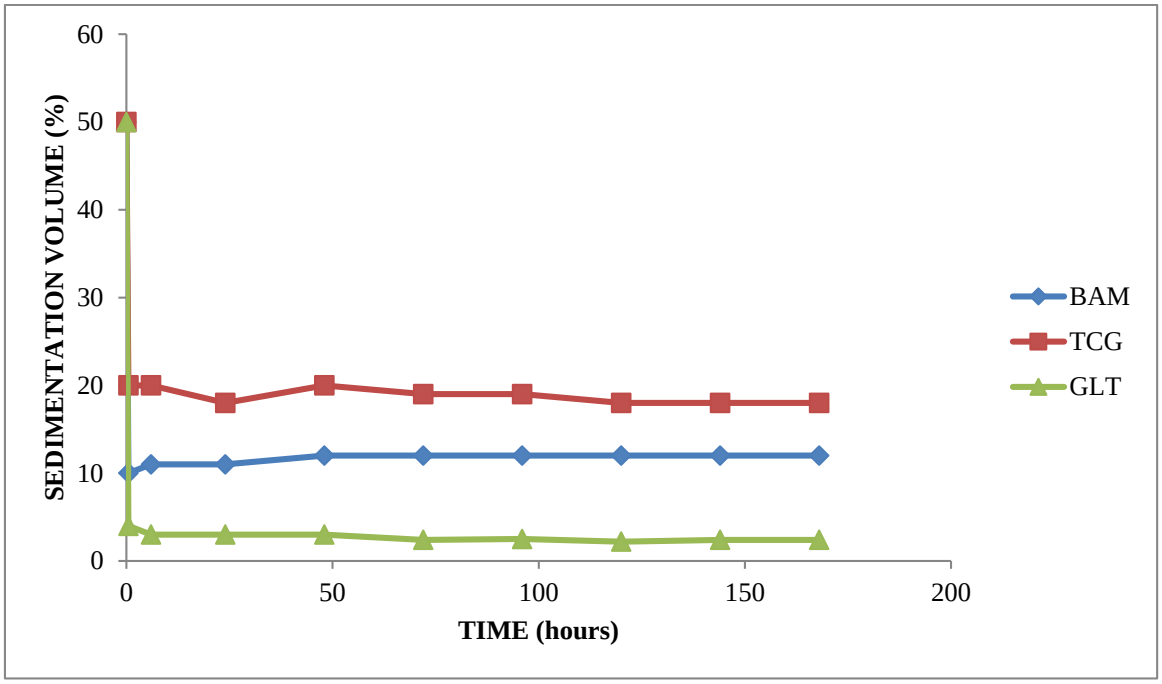

Figure 1: Representative plot of sedimentation volume against time at a concentration of 1.5% w/v suspending agent

The number of times taken to shake the suspension bottles before redispersion is presented in Table 4. There was no significant difference ($P > 0.05$) in the suspensions containing BAM and TCG. The low redispersion number could have been due to loose flocs formed by the sedimented suspensions. However, suspensions containing GLT with low sedimentation volume was observed to have significantly high ($p<0.05$) redispersion number. This could have been due to the formation of hard cake on settling, which made it difficult for redispersion. The suspension could have been a deflocculated one. Redispersion is an important aspect of the pharmaceutical quality of a dilute suspension since they tend to settle on standing (storage)[27,28]. Dose uniformity is dependent on the homogeneity of the suspension during administration; therefore, a suspension with low redispersion number will be desirable. The degree of flocculation was in the order GLT>BAM>TCG (figure 2).

Table 4: Redispersion number of suspensions at different concentartions

Suspending agent	Concentration(%w/v)	Redispersion number
	0.5	3
BAM	1.0	3
	1.5	3
	2.0	3
	0.5	3
TCG	1.0	3
	1.5	3

Suspending agent	Concentration(%w/v)	Redispersion number
GLT	2.0	3
	0.5	5
	1.0	5
	1.5	10
	2.0	15

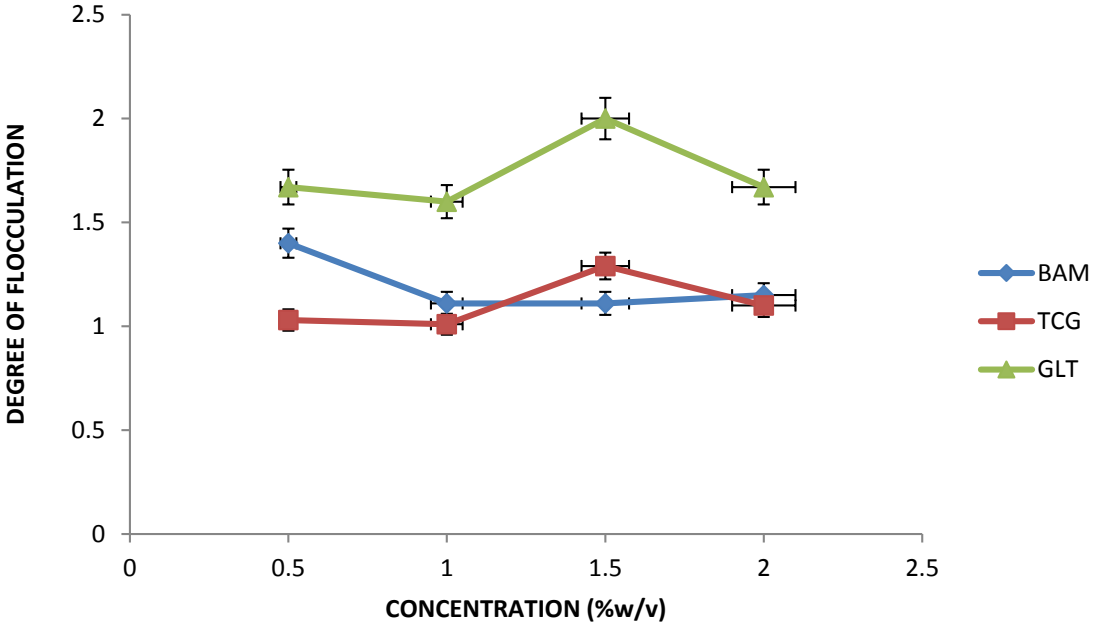

Figure 2: Plot of degree of flocculation against concentration

Conclusion

Formulated metronidazole suspension containing BAM mucilage has same flow rate as the formulations containing TCG. There were no changes in the viscosity of suspensions containing BAM with increase in concentration. There was no significant difference in the sedimentation volume of suspensions containing BAM and TCG. Therefore, BAM can be used as an alternative suspending agent in formulation.

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تقييم *Basella alba* L Mucilage كعامل تعليق في تعليق ميترونيدازول

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ملخص

في البحث عن بوليمرات طبيعية قابلة للتحلل وغير سامة لاستخدامها كسواغات في المستحضرات الصيدلانية، تم عزل الصمغ من جذع *Basella alba* L (BAM) وتقييمه كعامل معلق في معلقات الميترونيدازول بتركيزات مختلفة (0.5٪ – 2٪ وزن / ت) بالمقارنة مع تراجاكانث (TCG) ولثة الجيلاتين (GLT). تم تحديد الخواص الميكروميريتية لمسحوق الصمغ وتم تمييز معلق الميترونيدازول باستخدام معدل التدفق وعدد إعادة التشتت وحجم الترسيب واللزوجة ودرجة الحموضة. تم تحديد درجة التندف أيضًا. مسحوق BAM له خاصية تدفق جيدة مع الحد الأدنى من التورم. كان ترتيب معدل تدفق معلق الميترونيدازول BAM = TCG> GLT بينما كان ترتيب حجم الترسيب TCG> BAM> GLT. لم يكن هناك فرق كبير ($p> 0.05$) في عدد إعادة تشتت تركيبات BAM و TCG. كانت لزوجة التركيبات المحتوية على BAM و TCG بتركيزات 0.5٪ –1.0٪ وزن / حجم هي نفسها. تراوح الأس الهيدروجيني للمعلقات من 5 إلى 8. كانت درجة التلبد بالترتيب GLT> BAM> TCG. من النتائج التي توصلنا إليها، يمكن استخدام BAM كعامل تعليق بديل في صياغة التعليق.

الكلمات الدالة: *Basella alba*، معلق، عوامل تعليق، ميترونيدازول.

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تاريخ استلام البحث 2021/1/5 وتاريخ قبوله للنشر 2022/2/14.

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(2008/23.3/د)

عمادة البحث العلمي

المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

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

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

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

والله ولي التوفيق

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