Scientific Research Support Fund

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المجلة الأردنية في العلوم الصيدلانية

مجلة علمية عالمية متخصصة تصدر بدعم من صندوق دعم البحث العلمي والابتكار

Jordan Journal of PHARMACEUTICAL Sciences

Specialized International Refereed Journal

Issued by the Scientific Research Support Fund

This title is indexed in Scopus

مجلد (14) العدد (2)، حزيران، 2021

Volume (14) No. 2, June 2021

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 14, Number (2), *June* 2021

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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INTRODUCTION

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

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

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

Letter from the Editor-in-Chief

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

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

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

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

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

Prof Ibrahim Alabbadi
Editor-in-Chief

Editorial Commentary

Dear researchers,

Last year, 2020, was an exceptional year. A year that changed concepts and raised new challenges in several fields, and pharmacy practice was no exception. This was a year in which new vaccines were authorized for emergency use by the U.S. Food and Drug Administration after months of vaccine development which typically takes years to develop.

The pandemic has taken its toll on healthcare systems worldwide. Pharmacists were among the first responders to the public health needs. They took a role in the establishment and implementation of treatment guidelines for COVID-19 infection. Pharmacists worked collaboratively with multidisciplinary teams offering clinical and administrative services. Patient education in a time of information chaos and abundance of unreliable resources requires trustworthy professionals and pharmacists that can offer their knowledge and expertise to patients in different healthcare facilities.

One of the most memorable moments in 2020 was the introduction of new COVID-19 vaccines. Pharmacists have a role in administering vaccines and providing patient education about the vaccines. In addition, pharmacists have the vital responsibility of vaccine storage requirements and reconstitution procedures to ensure proper and safe administration. Universally, pharmacists in different drug authorizing bodies are participating in the authorization process of new COVID-19 vaccines. Their role is paramount in ensuring the safety and efficacy of vaccines. The vaccines that are surrounded by myths and conspiracy theories require science and scientists to clear the ambiguity and provide relief and comfort for individuals.

Research investigating numerous aspects related to the COVID-19 pandemic including treatment guidelines, manufacturing vaccines, safety and efficacy of the vaccines, and health-related outcomes is a sacred responsibility of the scientific community.

We at Jordan Journal of Pharmaceutical Sciences invite you to submit your work to our journal and together contribute to science everywhere.

Professor Linda Tahaineh
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Screening of commonly used plant extracts in Jordanian skin lightening folkloric recipes for their tyrosinase inhibitory activity: An *in vitro* study

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ABSTRACT

In Jordanian folkloric medicine, several medicinal plants–based recipes are used for skin lightening. Local recipes for skin lightening were collected and the tyrosinase inhibitory activity of the plants reported in these recipes, as a potential depigmentation mechanism was evaluated *in vitro* on both, mushroom and murine melanoma tyrosinase. The surveyed recipes included a total of 25 traditional medicinal plants belonging to 19 families. Kojic acid and licorice (*Glycyrrhiza glabra*) extract were used as positive controls. Thirteen extracts exhibited good mushroom tyrosinase inhibitory potential (>70%), and 7 extracts showed moderate tyrosinase inhibition activity (30-70%) while 5 extracts showed poor mushroom tyrosinase inhibitory activity (<30%).

Four of the tested extracts; *Juniperus communis* L. (Juniper), *Rosa indica* L. (Rose), *Amygdalus communis* var. *amara* L. (Bitter almond), and *Carthamus tinctorius* L. (Safflower) showed good inhibitory activity (>70%) against both, mushroom and melanoma tyrosinase enzymes that was similar or better than that of kojic acid. While, 6 tested extracts, obtained from *Raphanus sativus* L. (radish), *Juniperus communis* L. (juniper), *Petroselinum sativum* Hoffm. (parsely), *Salvia triloba* L. (sage), *Viola odorata* L. (garden violet), and *Mentha piperita* L. (mint), showed almost similar mushroom tyrosinase inhibitory activity as licorice extract (73.4%).

Tyrosinase inhibitory activities observed in many of the tested plant extracts validate their traditional use.

Keywords: Tyrosinase, Melanoma, Folkloric medicine, Skin lightening, Melasma.

1 INTRODUCTION

Skin hyperpigmentary disorders, such as melasma, freckles, and post inflammatory hyperpigmentation, are characterized by overproduction and accumulation of melanin [1]. They can have negative impact on subjects' psychosocial status since they are common on exposed areas of the face and the neck [1].The synthesis of skin pigment; melanin, takes place inside the melanocytes which reside in the basal layer of the epidermis. Melanin is formed by a complex pathway that initially involves two major reactions catalyzed by tyrosinase (a copper containing monooxygenase) [2]. Tyrosinase catalyses the

Received on 7/9/2020 and Accepted for Publication on 8/12/2020.

hydroxylation of L-tyrosine and the oxidation of the o-diphenol product L-DOPA (3,4-dihydroxyphenylalanine) to give rise to o-dopaquinone that is transformed into melanin through a series of reactions [2]. As a result of the key role, played by tyrosinase in melanin biosynthesis, most marketed skin lightening products use a tyrosinase inhibitor as the active ingredient (e.g., hydroquinone, kojic acid and arbutin). However, the use of these products is marred by safety and/or effectiveness concerns [3-5].

Thus, identification of new depigmenting agents, especially of plant origin, is an active research area that is reinforced by the belief that plant extracts have a superior safety profile to synthetic chemicals [6-8]. In cosmetic preparations many plant extracts such as *Morus alba* and *Glycyrrhiza glabra* have been used as depigmenting agents [9].

The use of skin-lightening products is a common practice among women living in Jordan [10]. In addition to its application in the treatment of hyperpigmentary disorders, the use of such products is reinforced by the beliefs that lighter skin tone plays a role in self-esteem, perception of beauty and youth as well as marriage and employment opportunities [10]. This is reflected in the fact that several plant-based skin lightening recipes are used in the Jordanian folkloric medicine.

In the present study, to accomplish the list of plants used in skin lightening recipes, the authors have interviewed major local herbalists in Amman and surveyed local folkloric medicine books. Then, the tyrosinase inhibitory activity of their aqueous extracts was evaluated to provide an evidence based justification for their folkloric use and to identify high potency extract(s) of local plants that might lead to the development of an effective skin lightening product for various hyperpigmentary disorders.

2. Materials and Methods:

2.1. Surveying and collecting folkloric skin lightening recipes:

Major herbalists (Attarins) in downtown Amman were interviewed and local folkloric medicine books were searched for local recipes used for skin lightening and for the treatment of hyperpigmentation. Some of these recipes are reported in Table 1. The plants reported in these recipes were purchased from the local market and identified by one of the authors (F. Afifi) using descriptive references and by comparison with the herbarium specimens of the Department of Biology, School of Science, University of Jordan. Voucher specimen were kept at the Faculty of Pharmaceutical Sciences, Hashemite University (FMSL1-FMSL26).

The scientific and common name of the reported plants, their families as well as the parts used in the recipes were determined and are summarized in Table 2.

2.2. Preparation of plant extracts:

Aqueous extracts of the plant parts specified in folkloric recipes were prepared by extracting each 5 grams of the powdered dried plant parts in 100 mL of distilled water at 60 ^{o}C for 2 hours. The resulting aqueous extracts obtained were filtered, stored in 50 ml centrifuge tubes (Jet-Biofil, Canada) and refrigerated at 4 ^{o}C until they were used in the *in vitro* tyrosinase inhibition assay.

2.3. Enzymatic assay of mushroom tyrosinase:

The effect of the prepared aqueous plant extracts on mushroom tyrosinase activity was determined spectrophotometrically using a previously published methodology after modifications and validation using both Kojic acid and licorice (*G. glabra*) extract as positive controls [9]. Mushroom tyrosinase (50 KU, Sigma, Aldrich) was aliquoted in potassium phosphate buffer (50 mM, pH=6.5) at final concentration of 500 U/ml and stored in -20 ^{o}C freezer until use. Twenty microliters (20 µl) of mushroom tyrosinase aliquot, and 60 µl of L-tyrosine (0.1 mg/ml) were incubated with different volumes (60, 120 µl) of the prepared aqueous plant extracts using 96-well plates. The final volume of each well was made to 220 µl with potassium phosphate buffer (50 mM, pH=6.5) and the plates were incubated at 37 ^{o}C for 20 minutes. The absorbance (Abs) at 490 nm was then measured using

microplate reader (680XR, Biorad, Bio-Rad Lab. Inc. USA). The same mixture without the plant extract was used as a control. Wells containing the plant extracts and mushroom tyrosinase without the substrate (tyrosine) were used as a blank to omit the effect of plant extract on absorbance. Licorice aqueous extract and Kojic acid (100µg/ml) in Phosphate Buffered Saline pH 7.2 (PBS, Euroclone, Italy) were used as positive controls.

The percent inhibition of tyrosinase activity was calculated as follows:

% Inhibition = (C - E) × 100/C ……. Equation (1)

Where C is the absorbance at 490 nm without plant extract, and E is the absorbance at 490 nm with plant extract. Results are shown in Table 3.

2.4. Cell Culture of murine melanoma:

The mouse (murine) melanoma, producing melanin; B16-F1 (ECACC 92101203) cells, were purchased from the ECACC (European Collection of Cell Culture) and cultured in DMEM (Euroclone, Italy) supplemented with 10% (v/v) fetal bovine serum (Euroclone, Italy), 1% L-glutamine (Euroclone, Italy) and 1% (v/v) antibiotic/antimycotic (100 units/ml, Sigma, Aldrich) at 37^{o}C in a humidified atmosphere with 5% CO_2. Cells were fed every other day until 80-90% confluency, cells were then harvested with 1X trypsin/EDTA (Euroclone, Italy) and lysed using 1% Triton X-100 (Promega, USA). Lysates were clarified by centrifugation at 13,000 rpm for 20 minutes and used in enzymatic inhibition assay after determining its protein content using Bicinchonic Protein Assay Kit (BCA) (Euroclone, Italy).

2.5. Enzymatic assay of murine melanoma tyrosinase:

The inhibition of melanoma tyrosinase in mouse melanoma cell lysate was performed as described previously with modifications [11-13]. Fifty microliters (50µl) of aqueous plant extract were added to a 96-well flat-bottom plate containing 50 µl of 4mM L-Tyrosine (Sigma, Aldrich) and 50 µl of 4 mM L-DOPA (Sigma, Aldrich). The plates were incubated at 37 ^{o}C for 10 minutes after which 50 µl of protein lysate containing equal amounts of protein (30 µg) in Phosphate Buffered Saline pH 7.2 (PBS, Euroclone, Italy) were added in each well. The final volume of each well was 200µl. The plate was then incubated at 37 ^{o}C for 60 minutes. The absorbance (Abs) at 490 nm was then measured using microplate reader (680XR, Biorad, Bio-Rad Lab. Inc. USA). The same mixture without the plant extract served as a control. Blank wells containing the extracts without protein lysate were used to omit the effect of plant extract on absorbance. Licorice aqueous extract and Kojic acid (100µg/ml) in PBS were used as positive controls. The percent inhibition of tyrosinase activity was calculated using Equation 1.

The enzymatic assay was repeated for each plant extract using only 100 µl of 2mM L-DOPA as the substrate without L-Tyrosine. The absorbance at 490 nm was then measured using plate reader. The same mixture without the plant extract served as a control. The percent inhibition of tyrosinase activity was calculated using Equation 1.

2.6. Stability study of selected plant extracts:

Mushroom tyrosinase inhibition assay was performed using freshly prepared plant extracts of *Crocus sativus, Lepidium sativum and Petroselinum sativum* and repeated after one month storage in the refrigerator (4 ^{o}C) in 50 ml centrifuge tubes (Jet-Biofil, Canada).

2.7. Data Analysis:

Enzymatic inhibition assays (mushroom and melanoma tyrosinase) were performed at least in triplicate at a minimum of 3 independent times. The percentage inhibition results were summarized in Table 3 as the Mean ± SD.

3. Results and Discussion:

A total of 25 traditional medicinal plants, (Table 2), belonging to 19 families were found in Jordanian folkloric recipes recommended to ameliorate skin hyperpigmentation or cause skin lightening.

The reported plants and plants found in the recipes mentioned in Table 1 were evaluated for their potential skin depigmenting effect by testing the tyrosinase

inhibitory activities of their aqueous extracts *in vitro* using both mushroom and murine melanoma tyrosinase.

Tyrosinase was targeted in the screening process as it is the rate-limiting enzyme in melanin production and its inhibition is one of the major strategies in developing new skin depigmenting agents [6]. Tyrosinase catalyses the first two steps of melanin production. It hydroxylates the amino acid L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), then oxidizes L-DOPA to produce L-DOPA quinone which is processed through several elaborate steps to produce melanin.

The aqueous extracts were used to imitate the traditional used practices in the folk medicines. The plant extracts that caused enzyme inhibition percentage of less than 30% were considered poor inhibitors and the ones that caused 30-70% inhibition of enzyme activity were considered moderate enzyme inhibitors, while good enzyme inhibitors were those extracts that caused more than 70% inhibition of the enzyme activity.

Table 3 summarizes the results of mushroom tyrosinase inhibition assay presented as percentage (%) of tyrosinase inhibition caused by two different volumes (120µl and 60µl) of the prepared aqueous plant extracts corresponding to the concentrations of 27.3 and 13.6 mg dry plant /ml. Out of the 25 plant extracts, only five extracts showed poor mushroom tyrosinase inhibitory activity (<30%) while seven extracts showed moderate tyrosinase inhibition activity (30-70%). The remaining thirteen extracts showed good mushroom tyrosinase inhibitory activity (>70%).

Table 3 also shows the results of murine melanoma tyrosinase inhibition assay, presented as percentage (%) of tyrosinase inhibition caused by 50µl of the prepared aqueous plant extracts corresponding to a concentration of 12.5 mg dry plant /ml. The murine melanoma tyrosinase inhibitory activity of the plant extracts was carried out under two substrate conditions, namely using L-tyrosine as a substrate and L-DOPA as a cofactor and using L-DOPA alone without the L-tyrosine. Out of the 25 plant extracts, nine extracts exhibited poor melanoma tyrosinase inhibition activity (<30%), and eight moderate inhibition activity (30-70%) while with six extracts good inhibitory activity (>70%) was observed.

On the other hand, the positive control *G. glabra* aqueous extract displayed 70-73% inhibition of mushroom tyrosinase and 43.9-62% inhibition of melanoma tyrosinase. The second positive control, Kojic acid (100µg/ml) caused 91% inhibition of the mushroom tyrosinase and 78% inhibition of melanoma tyrsoinase. Interestingly, four of the screened plant extracts; *Juniperus communis* (Juniper), *Rosa indica* (Rose), *Amygdalus communis* L. var. *amara* (Bitter almond), and *Carthamus tinctorius* (Safflower), exhibited inhibitory activities (>70%) against both mushroom and melanoma tyrosinase enzymes that were almost similar or even better than kojic acid inhibition (Table 3).

Most of the plant extracts had shown moderate to good mushroom tyrosinase inhibitory activities while only the listed first five plant extracts in Table 3 showed poor mushroom tyrosinase inhibition. Nevertheless, two of these five plants that showed poor mushroom tyrosinase inhibition potential exerted moderate to good inhibition of melanoma tyrosinase. The aqueous extract of *Nigella sativa* (black cumin) and *Rosmarinus officinalis* (Rosemary) showed 49.7% & 86.5% melanoma tyrosinase inhibition, respectively, and poor inhibition (< 30%) of the mushroom tyrosinase. Earlier, Subramanian and Sahithya (2016) revealed for the alcohol extract of *N. sativa* moderate inhibition (49.6%) of mushroom tyrosinase [14]. Similarly, researchers detected for the essential oil of *R. officinalis* poor mushroom tyrosinase inhibitory potential [15].

The aqueous extract of the seeds of sweet almond *A. communis* var. *dulcis* showed poor inhibition of the mushroom and melanoma tyrosinase. The aqueous extract of the hull of *A. communis* var. *dulcis* was previously studied and showed also poor tyrosinase inhibition potential [16]. However, the alcohol extract of the leaves of

the same plant was shown to possess moderate mushroom tyrosinase inhibition potential [17]. On the contrary, the aqueous extract of the seeds of bitter almond *A. communis* var. *amara* showed in the present study 100% inhibition of mushroom tyrosinase and 78.9% inhibition of murine melanoma tyrosinase. This was not reported previously for bitter almond based on literature search.

The aqueous extract of the peel of *Cucumis melo* (Muskmelon) showed in our study an inhibition of mushroom tyrosinase activity of 24.5-39.2% that agreed well with a previous report [18].

The aqueous extract of leaves of *Thymus vulgaris* (Thyme) resulted in a moderate inhibition of mushroom tyrosinase that was similar to the effect of its alcoholic extract reported in the literature [19]. The same extract showed an even higher inhibition of the murine melanoma tyrosinase (67.8-84%). It has been reported that *T. vulgaris* contain carvacrol; a monoterpenoid compound, that is shown previously to inhibit tyrosinase enzymatic activity in B16F10 mouse melanoma cells better than the kojic acid [20].

The aqueous extract of the leaves of *Origanum vulgare* (Origano) showed moderate inhibition of mushroom tyrosinase and good inhibition to murine melanoma tyrosinase. A novel phenolic glucoside; origanoside, has been previously isolated from *O. vulgare* aerial parts and its depigmenting potential was confirmed in both melanoma B16 cells and in animal study [21].

The aqueous extracts of *Curcuma longa* (Tumeric) roots and *C. sativus* (Saffron) stigmas/styles showed moderate inhibition of mushroom tyrosinase similar to that previously reported using the methanolic extracts of the same plants [22, 23].

The aqueous extract of the flower of chamomile, *Matricaria aurea,* is considered a commonly used herbal drink among Jordanians for various ailments [24]. The aqueous extract of this plant showed moderate inhibition to both, mushroom (31.9-53.8%) and murine melanoma tyrosinase (23.7-70.1%). This inhibition potential was shown previously for another chamomile species, *M. recutita* but not for *M. aurea* species [25].

Six tested plants showed almost similar mushroom tyrosinase inhibition potential as the positive control *G. glabra* (73.4%). Those were the aqueous extracts of *Raphanus sativus* (73.8%), *J. communis* (74%), *P. sativum* (75.3%), *Salvia triloba* (76.4%), *Viola odorata* (81.6%), and *Mentha piperita* (82.2%).

The propylene glycol extract of the roots of *R. sativus* showed previously 88% inhibition of mushroom tyrosinase comparable to the inhibition obtained in this study for the aqueous extract of the seed parts of the same plants [26].

For the ethanolic extract of the fruits of *J. communis* Jagal et al. (2017) have previously reported that this extract inhibits tyrosinase activity and lighten the UV-radiated skin of HRM-2 mice [27].

Petroselinum sativum, known as garden parsley and *Salvia triloba,* known as the East Mediterranean sage [28] are two of the most popular herbs in Middle Eastern kitchen have exhibited good inhibition of the mushroom tyrosinase and moderate inhibition of the murine melanoma tyrosinase (Table 3). Literature survey revealed no previous studies on the antityrosinase inhibitory activity for both commonly used culinary herbs.

The aqueous extract of garden violet (*Viola adorata*) showed 81.6% inhibition of the mushroom tyrosinase and 54.3% inhibition of the murine melanoma tyrosinase. Although similar inhibitory activities for the organic extract was reported, this is the first report on the inhibitory activity of the water extract of garden violet [29 30].

Mentha piperita (mint) is widely used as a common herbal tea among the Jordanians and classified as a culinary herb for many dishes. Its aqueous extract showed 82.2% inhibition of the mushroom tyrosinase. The plant essential oil was also previously reported to inhibit mushroom tyrosinase [31].

Rosa indica (Rose) showed 91.8 % inhibition of mushroom tyrosinase and 100% inhibition of murine melanoma tyrosinase. Again, no similar findings could be

retrieved from the literature survey.

The mushroom tyrosinase inhibitory efficacy of the methanolic extract of the seeds of nutmeg, *Myristica fragrans* (69%) was reported previously [9]. However, more potent inhibitory activity of the aqueous extract of the seeds (96.3%) was detected in the present study.

Although in an earlier study the methanolic extract of the seeds of *L. sativum* (Garden cress) did not exhibit any tyrosinase inhibitory potential, in the present study, for the aqueous extract of the same plant 99.1% inhibition of mushroom tyrosinase was observed [32].

The aqueous extract of the flowers of *C. tinctorius* showed 100% inhibition of mushroom tyrosinase and 84.9% inhibition of murine melanoma tyrosinse. The methanolic extract of another part of this plant; seeds, was previously reported to inhibit mushroom tyrosinase as well as reduce melanin content of B16 melanoma cells [33]. In addition, Carthamus yellow; the major pigment component extracted from *C. tinctorius*, showed both, tyrosinase inhibition potential and melanin reduction potential in B16F10 melanoma cells [34].

Fenugreek (*Trigonella foenum-graecum*) seeds are commonly used as a medicinal plant in Jordan for lactation deficiency and general weakness [24]. The findings of the current study indicated 100% inhibition of mushroom tyrosinase of the aqueous extract of its seeds. This depigmenting potential has been reported previously for the alcoholic extracts of the seeds of this plants which caused more than 50% inhibition of the mushroom tyrosinase [14]. Furthermore, its alcoholic extracts reported to possess anti-inflammatory properties as well as caused reduction in melanin synthesis in murine melanoma B16F1 cells [35].

Cicer arietinum (Chickpea) seeds is a key ingredient in hummus; a popular dip in Jordanian kitchens. Interestingly, Chickpea has been mentioned by the great traditional Persian scientists; Avicenna and Razi who mentioned the cutaneous benefits of this legume [36]. Although no previous studies were carried out for the antityrosinase inhibitory activity of chickpea, the results of the present study revealed 100% inhibition of the mushroom tyrosinase activity.

In addition to the evaluation of the antityrosinase inhibitory potential of the selected 25 plant species, the effect of storage in the refrigerator on the mushroom tyrosinase inhibition potential of the extracts was studied using three aqueous extracts as examples. Mushroom tyrosinase inhibition potential was tested at baseline for the freshly prepared extracts of these selected three plants and again thirty days after storage in the refrigerator. Results are shown in Table 4. Apparently the three tested plant extracts; *C. sativus*, *L. sativum* and *P. sativum* have preserved their mushroom tyrosinase inhibition potential. This finding supports the use of these aqueous plant extracts as potential stable and effective component in skin depigmenting formulations in the Jordanian traditional medicine.

4. Conclusion:

The use of most of the plant components in the collected recipes in ameliorating hyperpigmentary disorders and skin lightening appears to be substantiated by the enzyme inhibition studies. More than two third of the tested plant extracts exhibited moderate to good tyrosinase inhibition efficacy. Of the 25 extracts examined, five showed more than 70% inhibition of both mushroom and murine tyrosinase and only three plant extracts; *Cyperus esculentu* (Earth almond), *A. communis L. var. dulcis* (Sweet almond) and *Sambucus niger* (Elderberry) were poor inhibitors of both mushroom and murine tyrosinase.

Four of the screened plant extracts; *J. communis, R. indica, A. communis* L. var. *amara*, and *C. tinctorius* L., inhibited both mushroom and melanoma tyrosinase enzymes almost similar or even better than kojic acid.

Interestingly, the tyrosinase inhibition potential of the of *P. sativum* (parsley), *S. triloba* (sage), *A. communis* var. *amara* (bitter almond) and *R. indica* (rose), which exhibited good tyrosinase inhibition, has not been reported

previously based on the comprehensive literature survey.

The aqueous extracts of *C. sativus* (saffron), *L. sativum* (garden cress) and *P. sativum* (parsley) preserved their tyrosinase inhibitory properties for one month at 4 ^{o}C which indicates the stability of the active constituents and their potential to be incorporated into cosmeceutical formulations. Further *in vitro* and *in vivo* evaluation of the promising plant extracts are recommended.

Acknowledgment:

This project was funded by Abdul Hameed Shoman Scientific Research Support Fund, Amman, Jordan.

Conflicts of Interest:

The authors declare that they have no conflicts of interest.

Table 1: A number of Jordanian skin lightening folk recipes and direction for use. Plants in recipes are written in **Bold**.

No.	Components	Direction for use
1.	**Sage** extract and honey	Morning and evening
2.	**Common violet** and **elderberry** extracts	Morning and evening
3.	Boiled seeds of **radish**	Twice daily
4.	Extract of **parsley**	Apply at night and wash in the morning
5.	Extracts of **chickpeas**	Use as mask for 30 min and wash
6.	Peel of the **Muskmelon** and honey or olive oil	Apply to face for 20 minutes and wash with water
7.	Castor oil (*Ricinus communis*)	Twice daily for one month
8.	Powder of **Black cumin** and vinegar	Mix to form a paste, apply to skin for 30 min and wash
9.	Cucumber, **sweet almond, garden cress, Parsley**, Chicory	Orally and topically
10.	Juice of the **Muskmelon**	Once daily
11.	**Safflower** extract	Once daily
12.	Extract of **Fenugreek** seeds	Use cotton wool to apply to the skin twice daily
13.	**Garden cress** juice and honey	Twice daily
14.	Starch and **Saffron**	Mix together and apply to melasma
15.	Extract of **Origano**	Apply at night and wash in the morning
16.	Powdered **Muskmelon** peel and honey	Once daily for one week
17.	Oil of **sweet and bitter almonds**	Once evening for 3 days
18.	Castor oil	Once evening for 4 weeks
19.	Ground seeds of **nutmeg** mixed with honey	Mask for 1 hour
20.	Ground **earth almond** and honey	Mask for 1 hour
21.	**Mint** leaves extract	Apply and leave for overnight
22.	Juice of the **Muskmelon**	Mask for 1 hour
23.	Castor oil, **ground tumeric**, Vaseline and beeswax	Once in the evening for at least 4 weeks
24.	Castor oil, **earth almond**, Vaseline, beeswax	Once in the evening for at least 4 weeks

Table 2: The plants screened, their scientific names, families, Arabic names and parts used.

No.	Scientific name	Family	Common name	Arabic name	Parts used
1*	***Amygdalus communis* L. var. *dulcis***	Rosaceae	Sweet almond	Loz hilo	Seeds
2	***A. communis* L. var. *amara***	Rosaceae	Bitter almond	Loz mur	Seeds
3	***Carthamus tinctorius* L.**	Asteraceae	Safflower	O'sfur	Flowers
4	***Cicer arietinum* L.**	Fabaceae	Chickpea	Hummus	Seeds
5	***Crocus sativus* L.**	Iridaceae	Saffron	Z'afaran	Stigmas/Styles
6	***Cucumis melo* L.**	Cucurbitaceae	Muskmelon	Shomam	Peels
7	***Curcuma longa* L.**	Zingiberaceae	Turmeric	Curcum	Roots
8	***Cyperus esculentus* L.**	Cyperaceae	Earth almond	Hab alaziz	Fruits
9	***Glycyrrhiza glabra* L.**	Leguminosae	Licorice (positive control)	Aerq alsos	Roots
10	***Jasminum officinale* L.**	Oleaceae	Jasmin	Yasmin	Flowers
11	***Juniperus communis* L.**	Cuprassaceae	Juniper	Ar'ar	Aerial parts
12	***Lepidium sativum* L.**	Cruciferae	Garden cress	Rashad	Seeds
13	***Matricaria aurea* (Loeffl) Sch Bip**	Asteraceae	Chamomile	Babonej	Flowering heads, leaves
14	***Mentha piperita* L.**	Lamiaceae	Mint	Na'na	Leaves
15	***Myristica fragrans* L. Hout**	Myristicaceae	Nutmeg	Jozettib	Seeds
16	***Nigella sativa* L.**	Ranunculaceae	Black cumin	Alhaba alsouda	Seeds
17	***Origanum vulgare* L.**	Lamiaceae	Origano	Mardaqush	Leaves
18	***Petroselinum sativum* Hoffm.**	Umbelliferae	Parsley	Bakdonis	Leaves, stem
19	***Raphanus sativus* L.**	Brassicaceae	Radish	Fejil	Seeds
20	***Rosa indica* L.**	Rosaceae	Rose	Ward	Petals, flowers
21	***Rosmarinus officinalis* L.**	Lamiaceae	Rosemary	Iklil aljabal	Leaves
22	***Salvia triloba* L.**	Lamiaceae	Sage	Meramia	Leaves
23	***Sambucus nigra* L**	Caprifoliaceae	Elderberry	Bailasan	Flowers
24	***Thymus vulgaris* L.**	Lamiaceae	Thyme	Z'atar	Leaves
25	***Trigonella foenum-graecum* L.**	Leguminosae	Fenugreek	Holbe	Seeds
26	***Viola odorata* L.**	Violaceae	Garden violet	Banafsaj	Flowers

***FMSL1-FMSL26**

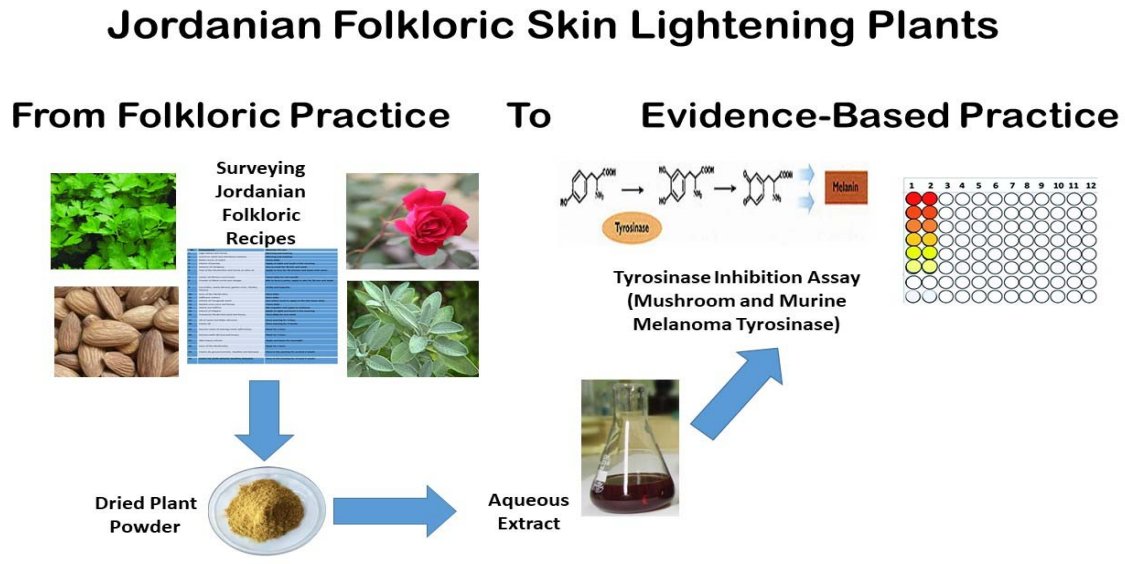
Table 3: Percentage mushroom tyrosinase inhibition at two different volume/concentrations of aqueous plant extracts (60 & 120 µl) and percentage of murine melanoma tyrosinase inhibition (using either L-tyrosine with L-dopa as substrate or L-dopa alone) by 50 µl of aqueous plant extracts found in folkloric recipes. Kojic acid (100µg/ml) & *Glycyrrhiza glabra L.* served as positive controls.

No.	Scientific name	Inhibition of mushroom tyrosinase %inhibition (± SD)		Inhibition of melanoma tyrosinase %inhibition (± SD) 50µl (12.5mg/ml)	
		120 µl (27.3 mg/ml)	60µl (13.6 mg/ml)	Tyrosine + DOPA	DOPA
1	*Cyperus esculentus* L.	0.4 (4.9)	6.5 (5.1)	11.1 (10.7)	10.2 (5.6)
2	*Nigella sativa* L.	12.4 (4.8)	16.8 (9.4)	49.7 (7.1)	1.1 (1.5)
3	*Rosmarinus officinalis* L.	19.4 (5.2)	28 (1.0)	86.5 (1.3)	93.0 (1.3)
4	*Amygdalus communis* L. var. *dulcis*	19.7 (4.6)	3.7 (9.1)	1.9 (3.2)	5.9 (5.3)
5	*Sambucus nigra* L.	21.6 (0.7)	0.1 (0.2)	ND	ND
6	*Jasminum officinale* L.	32.3 (14.9)	35.3 (9.1)	33.3 (3.9)	31.1 (7.0)
7	*Cucumis melo* L.	39.2 (10.6)	24.5 (3.8)	ND	ND
8	*Thymus vulgaris* L.	39.4 (5.6)	37.6 (2.6)	67.8 (3.7)	84.0 (3.6)
9	*Origanum vulgare* L.	41.4 (7.0)	16.5 (1.8)	71.9 (1.9)	83.2 (6.2)
10	*Curcuma longa* L.	42.7 (1.2)	44 (3.5)	17.3 (3.0)	13.9 (3.0)
11	*Crocus sativus* L.	50.1 (10.8)	7.8 (2.3)	9.9 (7.1)	4.9 (9.1)
12	*Matricaria aurea* (Loeffl) Sch Bip.	53.8 (1.1)	31.9 (9.4)	23.7 (13.8)	70.1 (4.9)
13	*Glycyrrhiza glabra* L.	70.4 (17.4)	73.4 (7.4)	62.3 (13.2)	43.9 (3.7)
14	*Raphanus sativus* L.	73.8 (8.0)	46.2 (3.7)	0.5 (0.2)	6.5 (5.5)
15	*Juniperus communis* L.	74 (0.7)	74.4 (7.2)	71.3 (39.8)	ND
16	*Petroselinum sativum* Hoffm.	75.3 (2.6)	35.5 (6.3)	2.6 (4.8)	56.4 (7.9)
17	*Salvia triloba* L.	76.4 (13.2)	59.6 (7.5)	2.5 (4.6)	61.7 (4.5)
18	*Viola odorata* L.	81.6 (9.3)	54.7 (5.5)	12.2 (2.7)	54.3 (18.6)
19	*Mentha piperita* L.	82.2 (16.4)	77.7 (12.2)	0.5 (0.3)	7.8 (4.7)
20	*Rosa indica* L.	91.8 (8.2)	82.8 (5.0)	100.0 (14.1)	80.5 (34.6)
21	*Amygdalus communis* L. var. amara	95.1 (7.0)	100 (6.3)	78.9 (15.8)	67.7 (6.5)
22	*Myristica fragrans* L. Hout	96.3 (1.1)	92.2 (3.1)	33.9 (5.9)	27.4 (4.3)
23	*Lepidium sativum* L.	98.5 (0.5)	99.1 (1.2)	6.5 (7.4)	10.4 (6.9)
24	*Carthamus tinctorius* L.	100 (25)	79 (11)	84.9 (13.3)	85.1 (15.1)

No.	Scientific name	Inhibition of mushroom tyrosinase %inhibition (± SD)		Inhibition of melanoma tyrosinase %inhibition (± SD) 50µl (12.5mg/ml)	
		120 µl (27.3 mg/ml)	60µl (13.6 mg/ml)	Tyrosine + DOPA	DOPA
25	*Trigonella foenum-graecum* L.	100 (13.5)	64.6 (9.1)	0.6 (0.3)	15.1 (1.4)
26	*Cicer arietinum* L.	100.5 (15.4)	71.8 (13.2)	0.62 (0.4)	0.33 (0.23)
27	Kojic acid (100µg/ml)	91.2 (1.6)		78.4 (0.9)	

Table 4: The stability of selected plant extracts (120µl) stored at 4ºC for one month.

No.	Scientific name	%Inhibition of mushroom tyrosinase (±SD) at two time points	
		0 time (freshly prepared)	After 1 month
1.	*Crocus sativus* L.	44.6±6.0	54.5±16.7
2.	*Lepidium sativum* L.	103.6±0.8	100.7±4.2
3.	*Petroselinum sativum* Hoffm.	62.9±0.5	53.3±11.7



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فحص المستخلصات النباتية الشائعة الاستخدام في وصفات تفتيح البشرة الشعبية الأردنية لنشاطها المثبط لإنزيم التايروسينيز (دراسة مخبرية)

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ملخص

في الطب الشعبي الأردني، تستخدم العديد من الوصفات القائمة على النباتات لتفتيح البشرة. تم جمع الوصفات المحلية لتفتيح البشرة وتم تقييم النشاط المثبط لمستخلصات النباتات المتواجدة في هذه الوصفات على كل من انزيم المشروم تايروسينيز وانزيم التايروسينيز من خلايا الميلانوما من الفئران كآلية عمل محتملة لتفتيح البشرة وازالة التصبغات.

شملت الوصفات الشعبية ما مجموعه **25** من النباتات الطبية والتي تعود الى 19 عائلة نباتية وتم استخدام حمض الكوجيك ومستخلص عرق السوس كضوابط إيجابية معروفة في تثبيط انزيم التايروسينيز.

أظهر ثلاثة عشر مستخلص نباتي تثبيط جيد لإنزيم المشروم تايروسينيز (تثبيط أكثر من 70% من نشاط الإنزيم)، وأظهرت 7 مستخلصات نباتية تثبيط معتدل لإنزيم المشروم تايروسينيز (تثبيط 30–70٪ من نشاط الإنزيم) في حين أظهرت 5 مستخلصات تثبيط ضعيف لإنزيم المشروم تايروسينيز (تثبيط اقل من 30٪ من نشاط الإنزيم) .

أربعة من المستخلصات المختبرة وهي **Juniperus communis (Juniper), Rosa. indica (Rose) Amygdalus communis var. amara (Bitter almond), and Carthamus tinctorius (Safflower)** أظهرت نشاطا مثبطا جيدا (أكثر من 70٪) لكل من إنزيم المشروم تايروسينيز وإنزيم التايروسينيز من خلايا الميلانوما من الفئران وكان تثبيطهم مماثلا أو أفضل من تثبيط حمض الكوجيك (الضابط الايجابي). وأظهرت ستة من المستخلصات النباتية نشاطا مثبطا لإنزيم المشروم تايروسينيز تقريبا مماثل لمستخلص عرق السوس (الضابط الايجابي) وهي

Raphanus sativus (radish), Juniperus communis (juniper), Petroselinum sativum (parsely), Salvia triloba (sage), Viola odorata (garden violet), and Mentha piperita (mint).

من الملاحظ ان نشاط العديد من المستخلصات النباتية في تثبيط كل من إنزيم المشروم تايروسينيز وإنزيم التايروسينيز من خلايا الميلانوما يدعم صحة استخدامها التقليدي في وصفات تفتيح البشرة وعلاج التصبغات.

الكلمات الدالة: التايروسينيز، الميلانوما، الطب الفولكلوري، تفتيح البشرة، الكلف .

تاريخ استلام البحث 2020/9/7 وتاريخ قبوله للنشر 2020/12/8.

Jordan Journal of Pharmaceutical Sciences, Volume 14, No. 2, 2021

Indirect Flow-Injection Spectrophotometric Determination of Some β-Lactam Antibiotics

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ABSTRACT

A simple and sensitive indirect spectrophotometric flow injection analysis (FIA) method for the determination of cefixime (CF), ceftriaxone (CFTR), cefotaxime (CFX) and cefuroxime (CFU) in pharmaceutical formulations and biological fluids has been suggested. Drugs under investigation are firstly oxidized by excess N-bromosuccinimide (NBS) in acidic medium, then the excess NBS is reacted with Rhodamine B (RB) to bleach its pinkish red color. Chemical variables and flow injection variables are all optimized to enhance reproducibility and sensitivity. The suggested procedure followed Beer's law over concentration ranges of 5 – 30 µg.ml-1 for all tested drugs. The method is successfully applied to the determinations of the drugs under investigation in different pharmaceutical preparations. Results obtained by the suggested method were in excellent agreement with those obtained by the formal HPLC methods.

Keywords: Cephalosporins, FIA, HPLC, Pharmaceutical Products, Human Plasma and Urine.

INTRODUCTION

The genus *Stereocaulon* Hoffm. (Stereocaulaceae, Lecanorales, Ascomycota) is an interesting genus from lichen which is found throughout the world. The morphology of the *Stereocaulon* genus consists of the crustose type primary thallus and fruticose type secondary thallus. The primary thallus in most species of *Stereocaulon* is disappeared at a very early stage of development. In the secondary thallus, there are several important parts such as pseudopodetia which show persistent phyllocladia (or phyllocladioid branchlets), apothecia as a sexual organ that contains spores, and in most species cephalodia which contain cyanobacteria (Nostoc, Rhizonema or Stigonema)[1].

Cefixime, ceftriaxone, cefotaxime and cefuroxime are kinds of cephalosporin antibacterial drugs. They are the second major group of semi-synthetic β- lactam antibiotics used in clinical medicine [1, 2]. They are used to treat infections induced by both gram-negative and gram-positive bacteria and interfere with the synthesis of vital structural parts of bacterial cell wall [3, 4].

Several analytical techniques for the determination of the drugs under investigation in biological and pharmaceutical preparations are described in literature. These include spectrophotometric [5-10], spectrofluorometric [11,12], chromatographic [13-16], capillary electrophoresis, electrochemical, potentiometry, voltammetry and flow injection analysis [17-20]. Chromatographic procedures are well-known and specific; however, they are time consuming and require sophisticated instruments. Equally, most batch spectrophotometric procedures require prior extraction of the colored product and take long reaction time for complete color intensity. Overcoming these drawbacks and automating the procedure are of current interest for the analysis of pharmaceutical compounds.

Received on 29/5/2020 and Accepted for Publication on 17/4/2021.

Because of the ability of the Flow Injection Analysis (FIA) method to conduct quick, accurate and precise analysis, it is of concern to evaluate its applicability in pharmaceutical products, plasma and urine samples. The automatic nature of FIA reduces the need for skilled and well- trained analysts to conduct the chemical analysis.

Experimental

Reagents and solutions

All chemicals used are in a pure grade and used without further purification as they have been obtained. During this entire work, distilled water is used. Hydrochloric acid solution (0.1 M) is prepared by diluting the calculated quantity of concentrated HCl with distilled water. Sodium hydroxide solution is prepared by dissolving the proper amount of NaOH in a total volume of 1 L with distilled water. N-Bromosuccinimide (NBS) solution ($1.0x10^{-3}$ M) is prepared daily by dissolving 0.0445 g of NBS (Merck) in a total volume of 250 mL with 0.1 M HCl. Rhodamine B (RB) solution ($2.0x10^{-5}$ M) is prepared by dissolving 0.0024 g of RB in a total volume of 250 mL with 0.1 M HCl.

Standard solutions

Standard solutions of the active ingredients were prepared by dissolving 0.01 g of the drug under investigation in a total volume of 100 mL of 0.05M NaOH. Standard solutions for linearity study are prepared by diluting the calculated volumes of the stock solution with 0.05 M NaOH.

Tablet, Capsule and Powders

The content of one capsule of the commercial product Suraxim (200 mg cefixime/capsule) is emptied in a 500 mL volumetric flask and hydrolyzed with 5 mL of 0.05 M NaOH for 30 min at 80°C. The concentration of cefixime in this solution is supposed to be 400 $\mu g.mL^{-1}$. The solution is used to prepare different concentrations within the linearity range by proper dilution with 0.05M NaOH. For tablets analysis, the content of one tablet of the commercial product Oraxim (250 mg cefuroxime /tablet) is emptied in a 500 mL volumetric flask and hydrolyzed with 5 mL of 0.05 M NaOH for 30 min at 80°C. The concentration of cefuroxime in this solution is supposed to be 500 $\mu g.mL^{-1}$. The solution is used to prepare different concentrations within the linearity range by proper dilution with 0.05 M NaOH. For powders, 0.01 g of the commercial product Ceftax powder (1000 mg of cefotaxime) and commercial product Samixon powder (1000 mg of ceftriaxone) is accurately weighed and transferred to 100 mL volumetric flask and then the procedure is continued as above.

Plasma and urine samples

A 500 µL of the drug under investigation (2000-ppm stock solution dissolved in water) is added to 1 mL drug-free plasma. After that, 0.5 mL of the 10% Trichloroacetic acid (TCA) is added. The mixture is vigorously mixed in a tube for 1 min and then centrifuged at 3000 rpm for 30 min to ensure complete protein precipitation. Next, 100-400 µL of the mixture is added to 10-mL volumetric flask and hydrolyzed with 5 mL of 0.05 M of NaOH for 30 min at 80°C. Urine samples are treated by the same procedure.

Apparatus

The suggested configuration of the FI system is shown in Figure 1. It's made up of two channels. A Varian DMS-100 UV-Visible spectrophotometer is used to perform all absorption measurements. Teflon tubing of 0.51 mm i.d. is utilized to build up the system. The sample solution is injected via a Rheodyne 6-way injection valve and combined with the carrier ($1.0x10^{-3}$ M NBS) in the first mixing coil (RC1). A home-made confluence point was used to ensure quick mixing of sample mixture with the reagent ($2.0x10^{-5}$ M RB) in the second reaction coil (RC2). A sample injection volume of 50 µL is used. The absorption of the color generated is tracked at 555 nm.

Procedure

A volume of 50 µL of the prepared sample solution is injected by a syringe into the carrier stream ($1.0x10^{-3}$ M NBS), pumped at a flow rate of 0.40 mL/min. The reagent ($2.0x10^{-5}$ M RB) was introduced downstream to ensure fast and sufficient mixing at a flow rate of 0.40 mL/min. After injection, when the maximum absorbance is reached, the valve is returned to the load position. Upon reaching the baseline, another sample slug is injected. The height of the absorbance peak is used for calibration.

Results and Discussion

Development of the Methods

The suggested FIA technique enables the target compounds to be quantified quickly and economically in pharmaceutical formulations without the need for time-consuming sample preparation steps. NBS have been extensively used as an analytical reagent [21-24]. In this study, it is found that NBS can oxidize the target drugs in acidic medium. In addition, it reacts Instantly with Rhodamine B (RB) in an acidic medium to fade out its color. Therefore, after the oxidation of drugs by NBS, the excess NBS is reacted with the RB. Figure 2 shows the absorption spectra of the reagents used and the reaction products. The decrease in the RB absorption (ΔA) is proportional to the drug concentration. Thus, the various parameters influencing the oxidation reaction, and hence the subsequent determination are optimized.

Influence of chemical variables

The effect of the acidity on the analytical signal, is considered over acidic and basic pH ranges. Various buffer solutions are being screened (acetate, citrate, borate and phosphate). No critical impact for the buffer type on the analytical signal is observed. All findings, however, stated that the reaction is better performed in acidic medium. Different concentrations of HCl are used as a solvent for both NBS and RB solutions. The maximum analytical signal is achieved when the carrier solution was 0.1 M HCl, which is used in all subsequent experiments (Figure 3).

The influence of changing RB concentration on the analytical signal is studied at different concentration of NBS as shown in Figure 4. Fixed volume (80 µL) of cefotaxime is used and injected into the NBS stream. For all NBS and RB combinations tested, best results are obtained when the NBS-to-RB concentration ratio was minimal. For instant, maximum signal was obtained at [NBS] = 0.2 mM and [RB] = 0.04 mM (NBS/RB = 5). However, the concentration of NBS must be high enough to react with both the drug and the RB. Therefore, a 0.7 mM NBS is selected to make sure that we have enough NBS to react completely with samples that contain high levels of the active ingredient (drugs under investigation). In addition, the base line stability is much better at 0.7 mM NBS. Based on these results, the concentrations of NBS and RB selected throughout this work are 0.7 mM and 0.04 mM, respectively.

Influence of FIA variables

The effect of the reagents flow rate is studied keeping other conditions constant, over the range 0.2 – 2.0 mL/min. In all cases, the same flow rate is used in both channels. The highest signals are obtained when the flow rate is 0.4 mL/min for each line (Figure 5). At higher flow rates, considerable decrease in the analytical signal is observed. This is because the reaction time will be lowered at greater pumping rates and therefore the reaction would not proceed to completion. At lower flow rates, the dispersion of the reaction product zone will increase leading to increased peak broadening and analysis time. Therefore, as a compromise between peak broadening, sensitivity, and sampling time, a total flow rate of 0.8 ml/min (0.4 ml/min for each line) is selected. At this pumping rate, the time for one injection is 45 seconds, and therefore the sample throughput is about 80 samples/hr.

The suggested FIA setup uses two reaction coils (Figure 2). The first coil (RC1), where the drug was oxidized by NBS, was changed over the 30 to 120 cm

range. When the length of the coil was increased to 50 cm, a significant rise in the analytical signal was noted and then began to decline (Figure 5). Longer coils led to peak broadening and a longer time to return to the baseline. The length of the first reaction coil (RC1) was therefore chosen to be 50 cm in order to ensure high sensitivity and high measuring rates. Similarly, when the second reaction coil (RC2) is changed from 30 to 120 cm, a substantial shift in the analytical signal is noted. Maximum analytical signals are acquired when RC2 is 60 cm (Figure 5).

Different lengths of the sample loop are mounted on the injector and tested to assess the impact of the injected sample volume on the analytical signal. As expected, an increase in the volume of the injected sample results in a peak height increase. As a result, measurement sensitivity could be enhanced by increasing the sample volume. However, by increasing the sample volume, the peak width and time for the signal to return to the base line are increased. A volume of 80µl, which ensure a reasonable sensitivity and sampling rate, is selected.

Evaluation of the method

Under the optimum conditions, the calibration curves for determining the studied drugs are constructed. Absorbance versus concentrations plots are straight lines. The linearity is excellent and Beer's law is followed for the drugs being investigated. Typical calibration data for drugs tested using the suggested FI technique are shown in Table 1. The precision of the measurements is high as reflected by the low RSD values (RSD is 1.15 % , n = 6). The detection limit (LOD) is determined as the analyte concentration resulting in a signal that is three times the blank standard deviation. Similarly, the limits of quantification (LOQ) is determined as the concentration of the analyte resulting in a signal that is ten times the blank standard deviation. The intra-day (within-day) precision is evaluated by the replicate analysis of two different concentrations of drugs within the linearity range at different time intervals. The inter-day (different days) precision is similarly evaluated on several days up to 3 days. Every day, a new calibration graph is constructed. The results in both cases indicated high precision, as the percent RSD did not exceed 3%.

Table (1): Data for the calibration graphs (n = 6) using the proposed FIA method

Parameter	Cefixime	Ceftriaxone	Cefotaxime	Cefuroxime
Linearity range ($mg.L^{-1}$)	5 - 30	5 - 30	5 - 30	5 - 30
Intercept (b)*	7.10×10^{-3}	1.60×10^{-2}	5.00×10^{-4}	1.01×10^{-2}
Slope (a)*	2.43×10^{-2}	2.82×10^{-2}	2.82×10^{-2}	2.55×10^{-2}
Corr. Coeff.(r^2)	0.999	0.997	0.994	0.997
LOD ($mg.L^{-1}$)	0.12	0.09	0.10	0.10
LOQ ($mg.L^{-1}$)	0.40	0.30	0.33	0.35

Applicability of the proposed FIA

The impact of prevalent excipients usually used in pharmaceutical formulations is studied to examine the applicability of the suggested FI technique to routine pharmaceutical evaluation. Synthetic mixtures containing different concentrations of drugs in the presence of more than 100 folds of common additives are prepared. The insoluble material is filtered off before injection. No interference from the additives usually present in commercially available products is observed and

recoveries between 103.9 and 99.3 % are achieved. An additional assessment of the suggested FI technique in pharmaceutical analysis is carried out by conducting recovery experiments from commercial formulations (Table 2). As shown in Table 2, the recoveries are excellent (100.3 – 108.8%) for all of the drugs tested, proving the potential of this method in pharmaceutical analysis.

Table 2: FIA and HPLC results for the analysis cefixime, ceftriaxone, cefotaxime and cefuroxime in pharmaceutical preparations.

Drug	Trade Name & Labeled Claim	Taken ($\mu g.ml^{-1}$)	%Recovery ± RSD, (n = 6)	
			FIA	HPLC
Cefixime	Suraxim (200mg/Capsule)	10	100.3 ± 1.3	101.4 ± 1.2
		20	103.0 ± 0.7	103.1 ± 1.7
		25	100.7 ± 0.5	102.3 ± 0.9
Ceftriaxone	Samixon (1000mg/ powder)	10	100.5 ± 1.2	100.0 ± 1.2
		20	105.3 ± 0.3	101.9 ± 1.8
		25	104.2 ± 0.6	103.6 ± 0.9
Cefotaxime	Ceftax (1000mg/ powder)	10	103.1 ± 0.5	102.9 ± 3.1
		20	102.9 ± 0.7	104.5 ± 0.6
		25	108.8 ± 0.4	106.9 ± 1.0
Cefuroxime	Oraxim (250mg/tablet)	10	100.9 ± 1.1	102.7 ± 5.2
		15	101.9 ± 2.9	102.6 ± 3.7
		20	104.8 ± 0.5	104.3 ± 4.2

Furthermore, the outcomes acquired by the FIA procedure are also compared with the outcomes acquired by the HPLC reference method [25] for the same sample set by means of 99% confidence level t- and F-tests. There are no significant differences between the two methods ' outcomes. obtained by the FIA method are statistically compared with those obtained by the official HPLC method. No significant differences in precision and accuracy between the outcomes of the two methods. It can therefore be concluded that, for routine assessment, the FIA technique has the benefit of being easier, faster and more practical.

Conclusion

A simple, sensitive and accurate FIA method for the analysis of Cefixime, ceftriaxone, cefotaxime and cefuroxime is proposed. The suggested method demonstrates excellent linearity, accuracy and reproducibility and is effectively implemented without interferences for the evaluation of the target drugs in pharmaceutical forms and biological fluids. The results

Acknowledgments

The financial support from Yarmouk University is gratefully acknowledged. The author would like to thank United Pharmaceutical Manufacturing Co. Ltd (Jordan-Amman), Jordanian Pharmaceutical Manufacturing (Jordan-Amman) and Al-Hikma pharmaceuticals (Jordan-Amman) for providing the standard and the excipients.

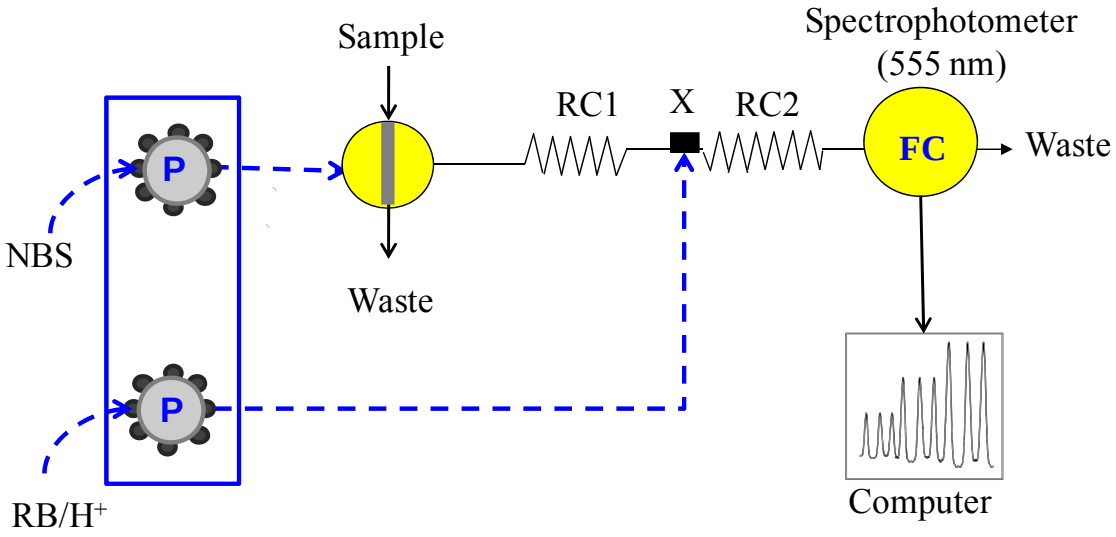

Figure 1: Schematic diagram of the proposed FIA system. P, peristaltic pump; RC1 and RC2 are the reaction coils; X, confluence point; FC, flow cell.

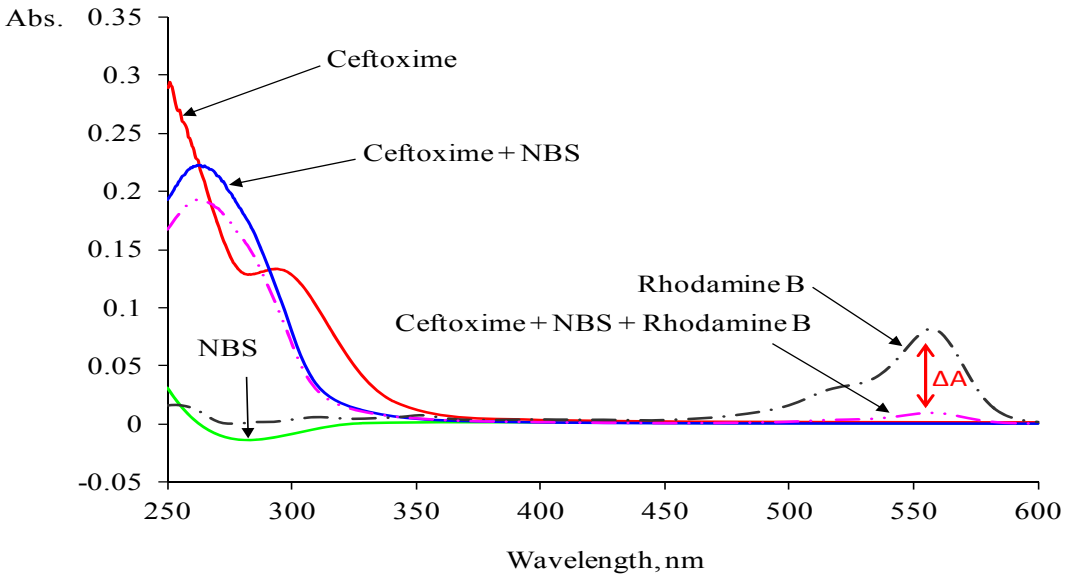

Figure 2: UV-Vis spectra for ceftoxime, NBS, RB, mixture of ceftoxime + NBS and mixture of ceftoxime + NBS + RB.

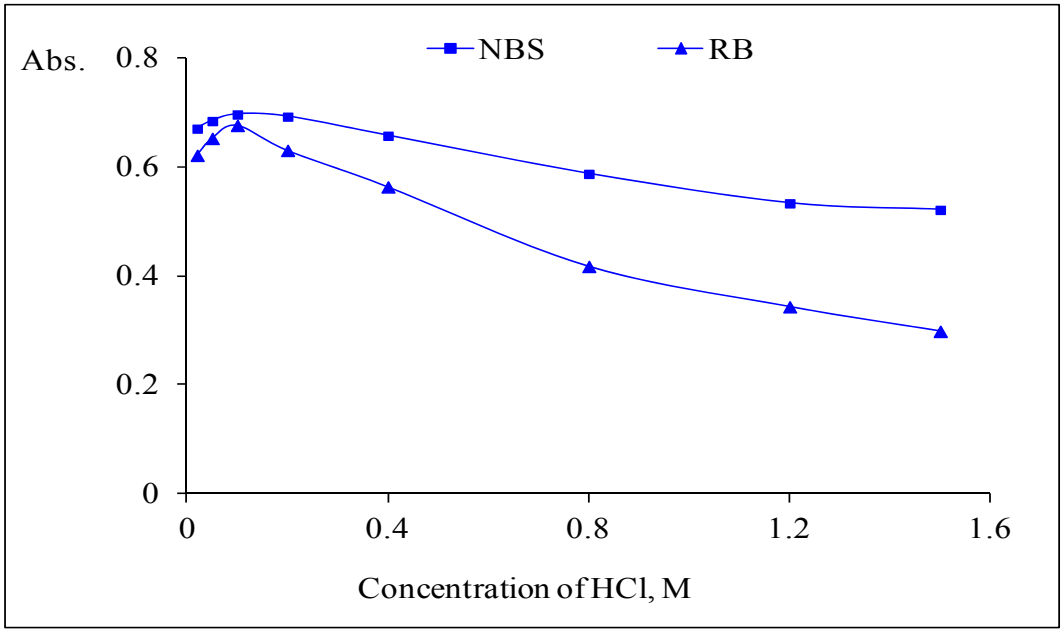

Figure 3: Effect of the reagents (NBS and RB) acidity on the analytical signal.

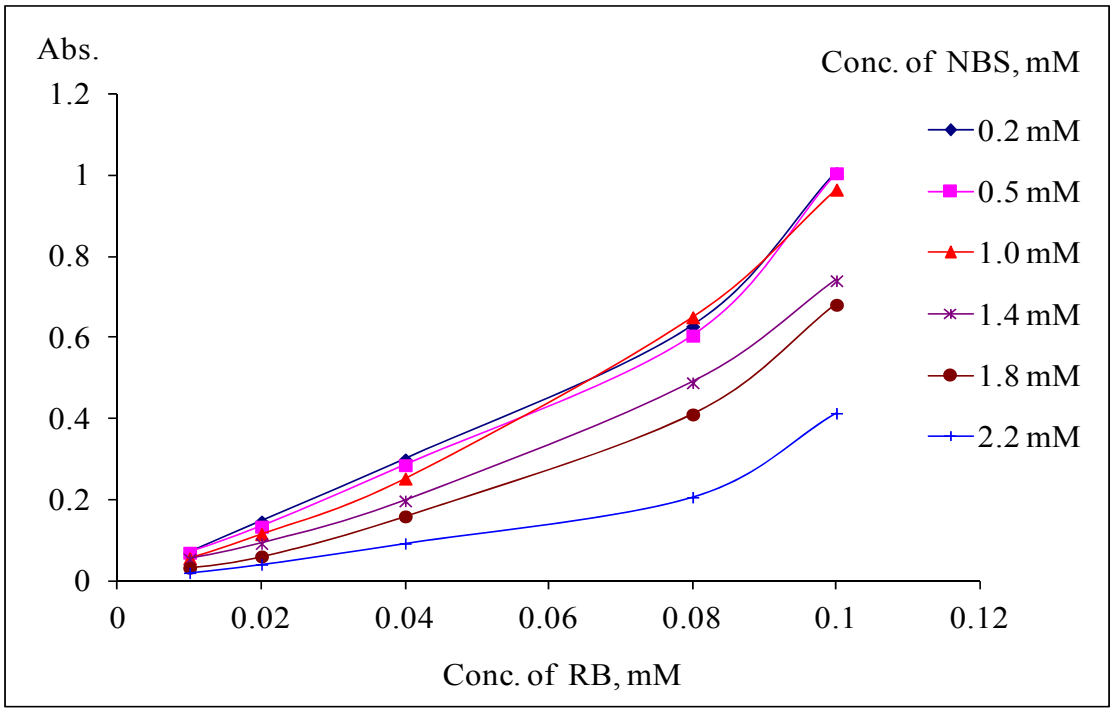

Figure 4: Effect of NBS and RB concentrations on the analytical signals.

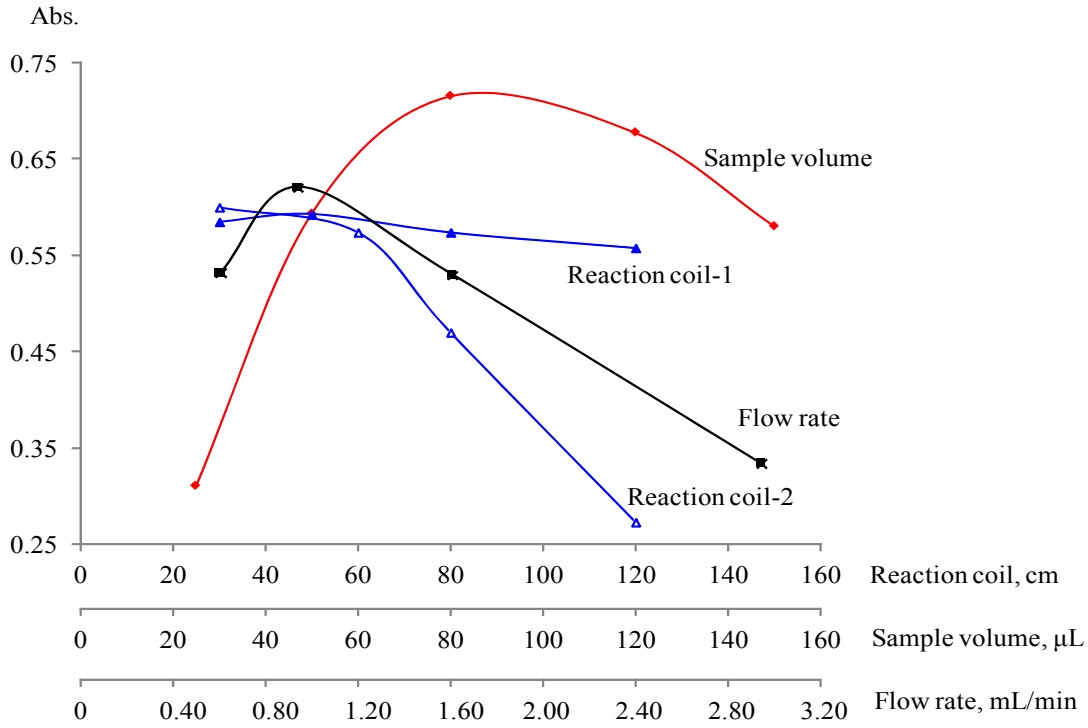

Figure 5: Effect of the flow rate, reaction coils (RC1 And RC2), and sample volume on the analytical signals.

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التحديد الطيفي غير المباشر لبعض مضادات بيتا لاكتام الحيوية بطريقة الحقن الجرياني

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ملخص

تم اقتراح طريقة جديدة بسيطة وحساسة لتقدير بعض مضادات بيتا لاكتام الحيويه في المستحضرات الصيدلانية والسوئل البيولوجية بطريقة الحقن الجرياني. تعتمد الطريقة الجديدة على اكسدة الدواء بكمية زائدة من **N-bromosuccinimide (NBS)** في الوسط الحمضي ثم مفاعلة ما تبقى مع صبغة **Rhodamine B (RB)** حيث يتسبب ذلك بقصر لون **RB** القرمزي. تم قياس الانخفاض في شدة اللون القرمزي عند الطول الموجي **555** نانوميتر. تم ضبط العديد من المتغيرات للحصول على اقصى قدر من الحساسية واعلى سرعة في التحليل. تم تطبيق الطريقة الجديدة بنجاح في تحديد الأدوية في المستحضرات الصيدلانية المختلفة. مقارنة النتائج التي تم الحصول عليها بالطريقة الجديدة بنتائج التحليل باستخدام الكروموتوغرافيا السائلة **HPLC** لنفس العينات اظهرت النتائج عدم وجود فروق معنوية ذات دلالة احصائيه بين نتائج الطريقتين.

الكلمات الدالة: مضادات بيتا لاكتام، التحليل بالحقن الجرياني، الكروماتوغرافيا السائلة، المنتجات الصيدلانية، بلازما الدم.

تاريخ استلام البحث 2020/5/29 وتاريخ قبوله للنشر 2021/4/17.

Jordan Journal of Pharmaceutical Sciences, Volume 14, No. 2, 2021

2D-QSAR modeling of novel pleconaril derivatives (isoxazole-based molecules) as antiviral inhibitors against Coxsackievirus B3 (CVB3)

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ABSTRACT

Because of their acute pathologies, the search for antiviral drugs for coxakievirus B3 (CVB3) is becoming an urgent and unavoidable necessity. In the present study, a series of Pleconaril derivatives (anti-CVB3 molecules) are subjected to 2D-QSAR study, in which the objective is the construction of a predictive model of new anti-CVB3 candidates that are more active than the ones studied. Two models are obtained by multiple linear regression ($R^2 = 0.893$; $Q^2_{CV} = 0.837$; $R^2_{test} = 0.778$) and multiple non-linear regression ($R^2 = 0.918$; $Q^2_{CV} = 0.784$; $R^2_{test} = 0.734$) showing very satisfactory results. Based on the obtained optimal QSAR models, novel Pleconaril derivatives are designed as new CVB3 inhibitors, showing remarkably improved inhibitory activity compared to the existing system. These results might be useful for advanced research in future experimental work.
Keywords: 2D-QSAR, MLR, MNLR, Coxakievirus B3, Pleconaril, Isoxazole, Antiviral.

INTRODUCTION

The heterocyclic compounds constitute a very important rate of organic compounds of natural or synthetic origins. These compounds have a wide range of biological activity [1-6].

The derivations of the isoxazole form a particular and interesting class of heterocyclic compounds with five links, due to their availability and their biological and pharmaceutical activities which are important and efficient such as the anti-inflammatory, analgesic, antiviral, antioxidant, anticancer and antimicrobial activities [7-12].

Coxakievirus B3 (CVB3) is a class of viruses belonging to the Picornaviridae family, one of the oldest and most diverse families of viruses and more specifically to the group of enteroviruses [13]. These are genera that have been detected in both humans and animals [13].

Received on 29/5/2020 and Accepted for Publication on 17/4/2021.

Like most viruses, CVB3 is transmitted by two main routes: aerosol and fecal-oral routes [14-15] . They target all age groups and precisely new infants and children under 15 years of age [14-16]. As soon as reach their target, CVB3s cause infections of the central nervous system, respiratory infections, mucocutaneous, muscular, and digestive disorders [17-18].

These infections occur after a set of processes that make up the replication cycle that begins with the entry of the virus after attaching itself to specific receptors. A process is then triggered to release viral RNA into the cytoplasm of the cells. After the protein expression and replication is followed by the assembly, new virions are then released and transmitted through the bloodstream to reach different destinations [15].

CVB3s are small viruses (diameter about 30nm), non-enveloped, their capsid has icosahedral symmetry. It consists of 60 copies of four proteins VP1, VP2, VP3, and VP4. The VP1, VP2, and VP3 proteins are located at the

outer surface of the capsid while VP4 is located at the inner surface [19]. Several studies have confirmed that the VP1 protein has been involved in the process of binding to the receptors of the cells [20,21].

Inside VP1 and just below the bottom of the canyon, there is a hydrophobic pocket that contains fatty acid [22]. This pocket contributes to the stability of the virus and also represents the binding site for antiviral molecules. These molecules displace the lipid presented in the hydrophobic pouch, then blocking the decapsidation process [23] so expression and replication of new viruses.

The main objective of our study is to model the inhibitory effect of a series of 28 Pleconaril derived compounds (isoxazole-based molecules) against CVB3, using statistical tools Principal Component Analysis (PCA), Multiple Linear Regression (MLR), Multiple Non-Linear Regression (MNLR). The current study includes four main steps: the selection of the dataset, generation of molecular descriptors, construction and validation of predictive models and finally proposing new candidate compounds that are more active than the studied ones.

MATERIAL AND METHODS

1- Database

In the present study, we selected 28 Pleconaril derivatives (Table 1) with activity values reported in the literature [24]. The activity expressed by IC_{50} is defined as the concentration necessary to decrease the initial rate of effect of CVB3 with a percentage of inhibition 50%. For modelling purposes, the activity was expressed in pIC_{50} ($pIC_{50}= -\log_{10}(IC_{50})$). To build and validate our QSAR model, the data set was divided into two sets: 23 molecules constituted the training set and the remaining 5 molecules constituted the test set. The division of the data set was performed by random selection.

2- Data processing

A wide variety of molecular descriptors were computed using Gaussian 09W, Chemoffice2016, MarvinSketch and ACD/ChemSketch 2019.13 to predict the correlation between these descriptors and the activity of the molecules studied (Tables 2 and 3) [25-28].

The electronic descriptors were obtained after optimization of the studied molecules using the 6-31G(d,p) [29] base of the Lee-Yang-Parr three-parameter Becke function (B3LYP) [30], which is a kind of density functional theory DFT method [31].The calculation of the descriptors started by drawing the molecules in GaussView 5.0 [32], then opening these structures in Gaussian 09W, and then executing the optimization (the calculations).

3- Selection of descriptors

The QSAR model is supposed to be simple and understandable, the descriptors chosen must be meaningful and interpretable and it must have the fewest parameters to explain the activity [33]. In order to reduce their number of descriptors, the highly correlated descriptors are removed based on the results of the Principal Component Analysis (PCA). A random procedure was used for the selection of the compounds of the learning set and the test set.

4- Principal Component Analysis (PCA)

PCA is an essentially descriptive statistical method, which aims at extracting the maximum amount of information contained in the compounds from the dataset [34] . The results of the PCA analysis are used to select the MLR input data. Thus, initially, we eliminated all descriptors with small (non-significant, $r \leq 0.3$) correlations with the dependent variable (pIC_{50}). To reduce redundancy in our data matrix, the descriptors that are highly correlated ($r \geq 0.9$) and have a low value of the correlation coefficient concerning the dependent variable were excluded (Table 4).

5- Multiple Linear Regression (MLR)

It is one of the most transparent modeling methods whose the prediction is easily achievable [35]. The principal of MLR is based on the fact that the dependent variable Y (Activity/Property) depends linearly on the different variables (the descriptors), according to the relation:

$$Y = a_i + \sum_{i=0}^{n} a_i X_i$$

Y: the dependent variable; X_i: the independent variables; n: the number of independent variables; a_0: the constant of the model equation; a_i: the coefficients of descriptors in the model equation.

6- Multiple non-Linear Regression (MNLR)

Despite its transparency, linear regression has certain disadvantages. It is deficient in the detection of non-linear dependence (exponential, logarithmic, polynomial,). To make up for this deficiency, a non-linear regression (NLR) is performed.

In our work we used the second-degree polynomial model, based on the descriptors proposed by the linear model, as shown in the following equation:

$$Y = a_0 + \sum_{i=1}^{n} (a_i X_i + b_i X_i^2)$$

Y: is the dependent variable; X_i: the independent variables; n: the number of explanatory variables; a_0: the constant of the model equation; a_i and b_i: the coefficients of descriptors in the model equation.

7- Model Validation

The stability and robustness of the model must be verified before using it for prediction [36]. There are two types of validation: internal and external validation.

7.1- Internal validation

Internal validation techniques include cross-validation, which consists of extracting one or more compounds (LOO) from the training set and reconstructing the model to calculate the activity (or property) of these compounds [37]. The correlation coefficient between the predicted and observed activities of the extracted compounds must be greater than 0.5 $(Q^2_{CV} > 0,5)$ [38].

Another internal validation technique; Y-Randomization is performed to assert that the model is not due to chance. It consists of randomly disorganizing the activities/properties N times (e.g. 100) and the columns of the descriptors remain unchanged [39]. This gives N models with specific statistical characteristics. These N models must have low performance [39].

In general, these internal validation techniques allow the evaluation of the robustness and the stability of the QSAR/QSPR model parameters concerning the molecules of the training set. However, they do not demonstrate in any way the predictive power of the models, which is why an external validation of the model is required [38,40].

7.2- External validation

The external validation consists of predicting the activity/property of the compounds constituting the test set, this validation is characterized by the parameters R^2_{test}, R_{test}, and $Q^2_{cv(test)}$. Several recent studies have shown that these parameters are insufficient to verify the predictive power of the model [41,42]. Therefore other parameters should be calculated [42].

- Metrics r^2_m:

To better indicate the predictability of the model, the metrics introduced by Roy *et al* [43] can be exploited by r^2_m which is defined by the following equations:

$$\bar{r}^2_m = \frac{(r^2_m + r'^2_m)}{2}$$

$$\Delta r^2_m = |r^2_m - r'^2_m|$$

Where $r^2_m = r^2 \times (1 - \sqrt{(r^2 - r^2_0)})$ and

$$r'^2_m = r^2 \times (1 - \sqrt{(r^2 - r'^2_0)})$$

Tropsha and Golbraikh criteria:

Golbraikh and Tropsha [41] proposed a set of parameters to determine the external prediction of the QSAR model (Table 7).

8- Applicability domain

This part of the analysis is explicitly requested [44]. The domain of applicability (DA) defines the zone in which a compound can be predicted with confidence. Indeed, the QSAR/QSPR built model is not intended to be used outside this domain [45].

There are several methods for determining the scope of application of a QSPR/QSAR model, including the "leverage" method. This method is based on the variation of the standardized residuals of the dependent variable according to the leverages. If a compound has a leverage that exceeds the threshold h*=3(k+1)/N (where k is the number of descriptors and N the number of molecules constitutes the learning set), this compound is considered as an influential compound on the model developed [46].

Another recently developed approach [47] gave comparable results to the "leverage" method. The algorithm of this new approach is as follows:

-All the descriptors appearing in the developed model (for training and test set compounds) are standardized using the following formula:

$$S_{ki} = \frac{|X_{ki} - \bar{X}_i|}{\sigma_{ki}}$$

k: total number of compounds (Training set and test set).

i: total number of descriptors.

X_{ki}: Standardized descriptor i for compound k.

$\bar{X}_i$: Mean value of the descriptor Xi for the training set compounds only.

σ_{ki}: standard deviation of the descriptor Xi for the training set compounds only.

The above-calculation should run for all descriptor values present in the model.

- Thereafter, one needs to compute the S_{ik} values. If $S_{ik(max)} < 3$, then that compound is not an X-outlier (if in the training set) or is with in applicability domain (if in the test set).

- If $S_{ik(max)} > 3$, then one should compute $S_{ik(min)}$. If $S_{ik(min)} > 3$, then the compound is an X-outlier (if in the training set) or is not with in applicability domain (if in the test set).

- If $S_{ik(max)} > 3$ and $S_{ik(min)} \leq 3$, then one should compute $S_{new(k)}$ from the following equation:

$$S_{new(k)} = \bar{S}_k + 0.28 \times \sigma_{S_k}$$

$S_{new(k)}$: S_{new} value for the compound k.

$\bar{S}_k$: Mean of $S_{i(k)}$ values of the compound k.

σ_{S_k}: Standard deviation of $S_{i(k)}$ values of the compound k.

RESULTS AND DISCUSSION

A series of 28 molecules (Table 1) derived from pleconaril were studied to determine a quantitative relationship between the structure of these molecules and their antiviral activity (pIC_{50}) against coxsackievirus B3 (CVB3).

1- Multiple linear regression (MLR)

The resulting model is a linear combination of three descriptors: n (Refractive Index), E_T (Total Energy), and μ (Chemical potential).

The model equation is as follows:

$$pIC_{50} = 16,826 - 11,468.n + 1,728.10^{-4} E_T - 3,722.\mu \quad (1)$$

The activity values (pIC_{50}) calculated by equation (1) of the MLR model are given in Table 9.

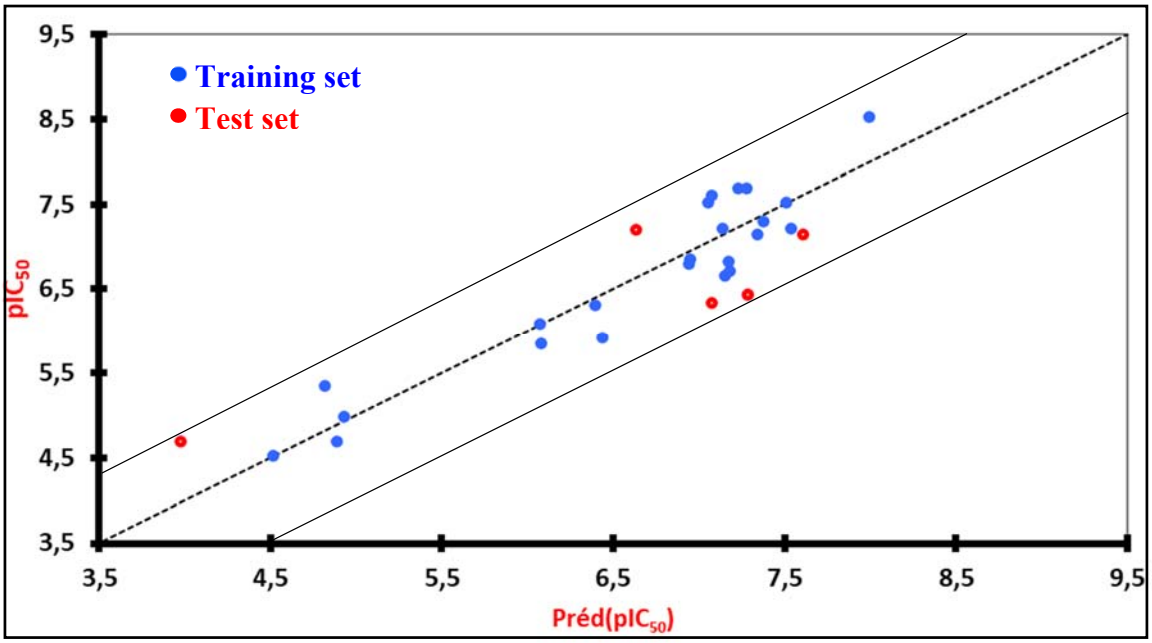

Figure 1: Graphical representation of calculated and observed activity for MLR

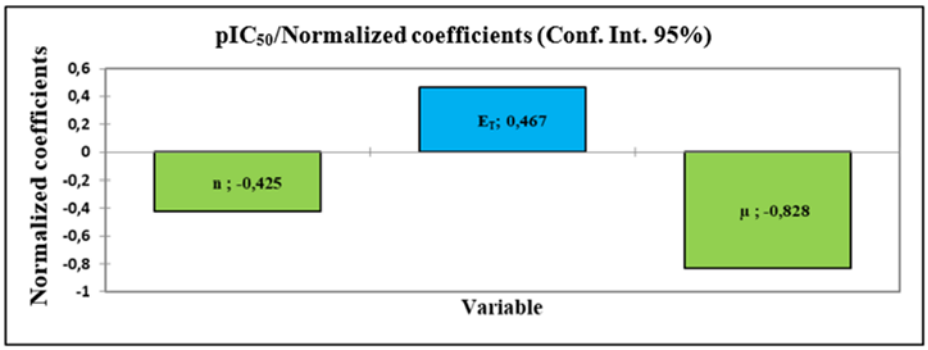

Figure 2: The relative contributions of the three descriptors for MLR

The relative contributions of the three descriptors are shown in Figure 2.

The Refractive index and chemical potential contributed negatively while total energy contributes positively.

The contribution of the refractive index and total energy were comparable, while the contribution of chemical potential was significant.

The value of the variance inflation factor (VIF) is calculated according to the equation:

$$VIF = \frac{1}{1 - r^2}$$

Where r^2 is the coefficient for determining the multiple regression equation between the model descriptors. If the VIF is equal to 1, no intercorrelation exists for each variable and if it is between 1 and 5, the constructed model is acceptable and

if it is greater than 10, the associated model is unstable and verification is required.

The VIF value of each descriptor used in the model (Table 5) is less than 5 and close to 1, which is an indication of the slight correlation of each descriptor to the other two.

The value of t for a descriptor is related to its statistical significance. High absolute values of t indicate that each regression coefficient is significantly larger than the associated standard deviation.

The probability P of t of a descriptor gives its statistical significance when is combined with other descriptors in the model (i.e., provides information on the interactions between descriptors). Descriptors with probability values of t below 0.05 are considered to be statically significant for a given model, i.e., their influence on the dependent variable (the response) is not due to chance.

The values of the probabilities of t for the three descriptors of the developed model (Table 6) were all lower than 0.001, indicating that the chosen descriptors are highly significant.

Table 7 contains the various calculated parameters and the criteria that must be met for the model to be acceptable.

The obtained results have shown a good correlation between the three selected descriptors and the studied activity, which is characterized by satisfactory statistical parameters. The good quality of the fit, the robustness, and the predictive power of the model has been confirmed by the high values of R^2, R^2_{adj}, $Q^2_{CV(LOO)}$, F, and low error values (MSE, RMSE).

The results obtained were very satisfactory and reflect the reliability of our model.

2- Multiple non-linear regression (MNLR)

We also used multiple nonlinear regressions to make up for the nonlinearity that is missing in multiple linear regression (MLR) and thus improve its results.

The method was applied to the same learning set and descriptors used in multiple linear regression.

The model equation obtained is as follows:

$$pIC_{50} = 195,37 - 180,41.n + 8,03.10^{-1}.E_T + 12,85.\mu + 54,52.n^2 + 6,46.10^{-2}.E_T^2 + 2,10.\mu^2 \quad (2)$$

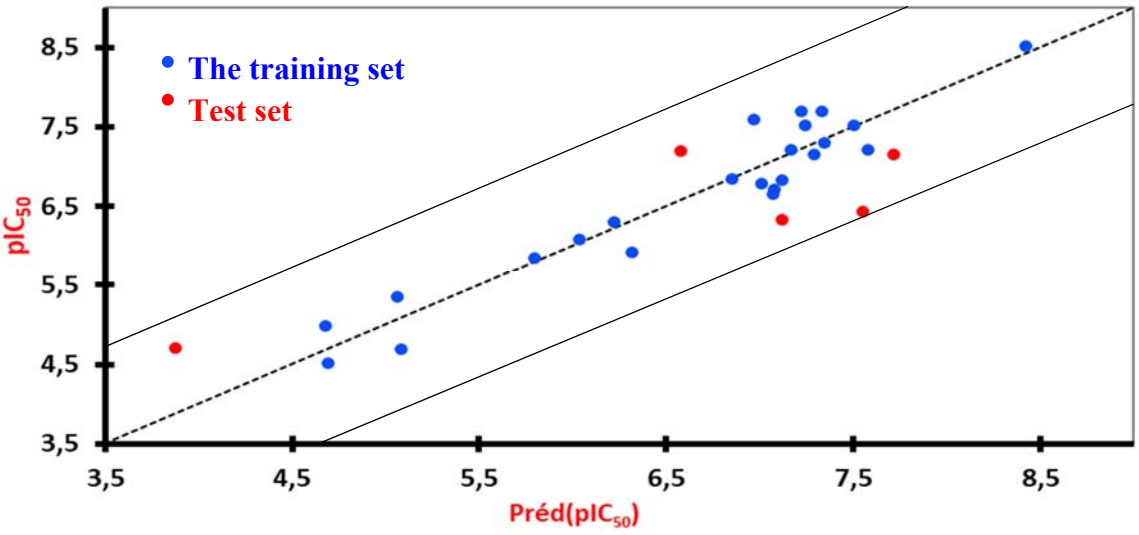

Figure 3: The correlation between observed and calculated activities for MNLR

The activity values (pIC_{50}) calculated by equation (2) of the MNLR model are given in Table 9.

The following table contains the calculated parameters for the model.

The overall obtained results are very satisfactory and reflected the reliability of the model obtained.

The good fit, robustness, and predictive power of the model have been confirmed by the high values of R^2, R^2_{adj}, $Q^2_{CV(LOO)}$, F, and low error values (MSE, RMSE).

3- Randomization test

To avoid chance correlations and validate the MLR and MNLR models built, randomization testing was applied, and 100 models were developed for both MLR and MNLR.

Figures 4 and 5 show the low values of $Q^2_{CVLOO(Rand)}$ and R^2_{Rand} obtained for the two models MLR and MNLR. The results confirm that the obtained models were not due to chance correlation.

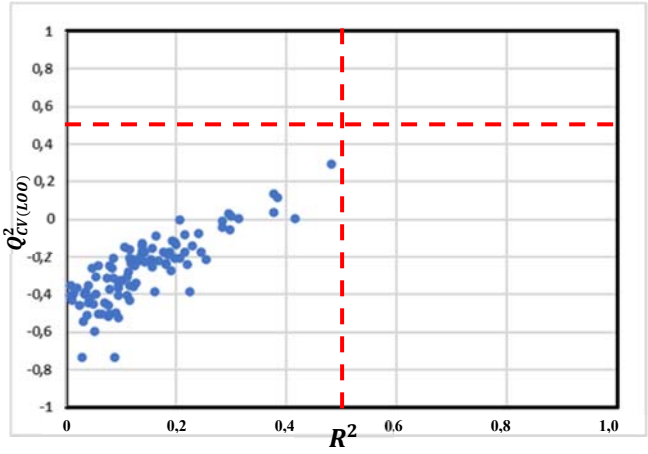

Figure 4:Y- Randomisation plot for MLR

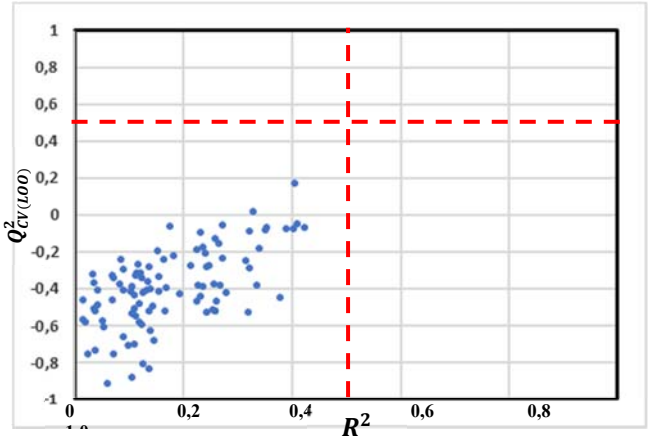

Figure 5:Y- Randomisation plot for MNLR

Figures 4 and 5 show the low values of $Q^2_{CVLOO(Rand)}$ and R^2_{Rand} obtained for the two models MLR and MNLR. The results confirm that the obtained models were not due to chance correlation.

4- Applicability domain

Both Figures 6 and 7 showed the standardized prediction errors as a function of the lever values (hi) and the S_{new} values of the molecules in the two training and test sets.

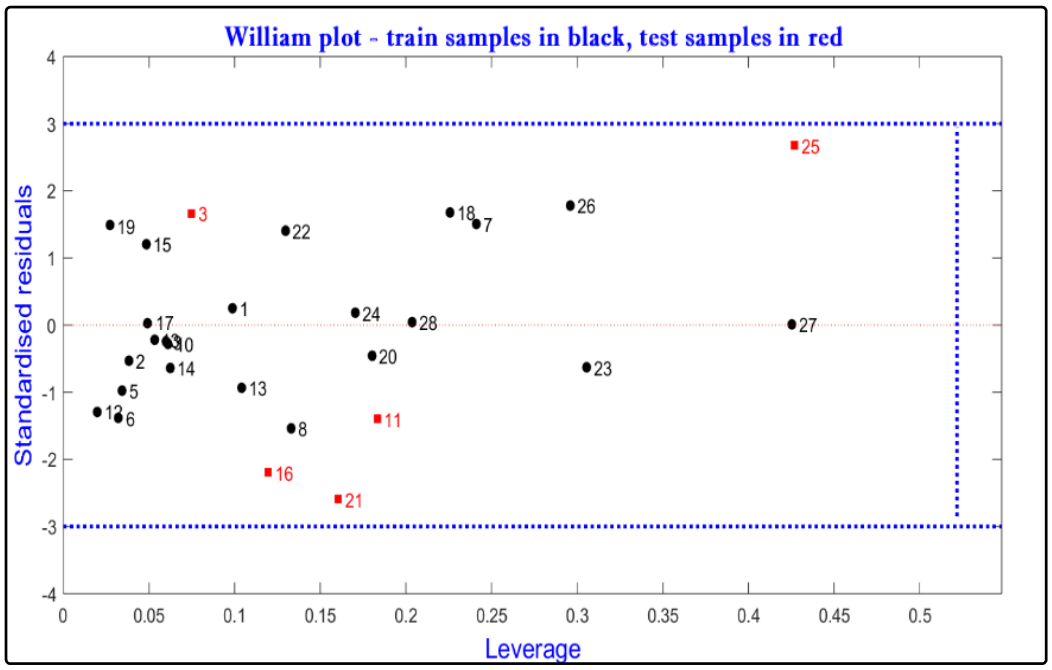

Figure 6: Williams plot to evaluate the applicability domain of MLR model (h*=0.522 and residual limits=±3).

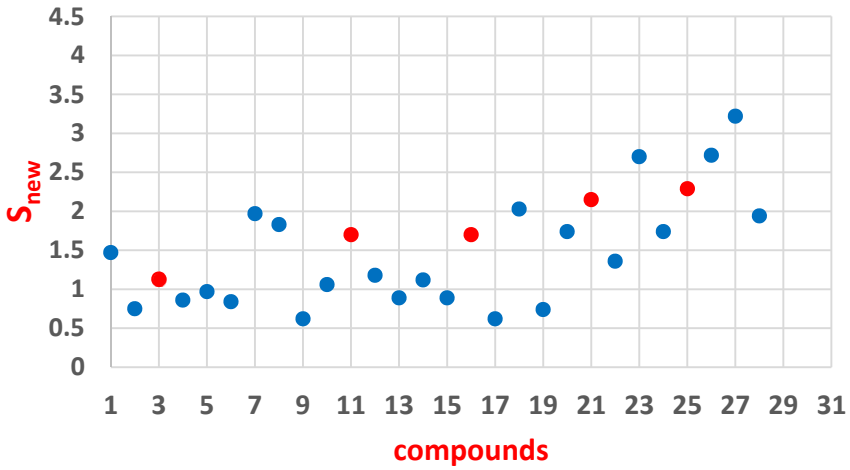

Figure 7: The S_{new} values of the test training and test sets

All the tailings are found to be in the range of (±3SD) (horizontal lines) and all compounds had levers $h_i < h^* = 0.522$ (Figure 6) and had a S_{new} of less than 3 (Figure 7), so there are no outliers for both training and testing sets, meaning that the model has a good predictive capability.

PERFORMANCE COMPARISON OF TWO MODELS MLR AND MNLR

A comparison of the results obtained by the two models was made (Figure 8).

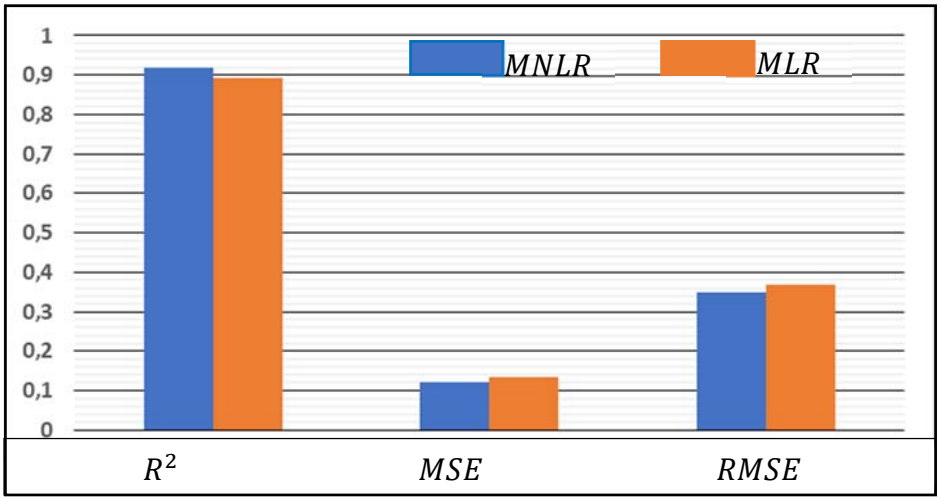

Figure 8: Performance comparison of the two models MLR and MNLR

The two established linear (MLR) and non-linear (MNLR) models generally performed well. Based on this comparison, it can be said that the non-linear model gave slightly better results than the linear model, this implied that the studied activity has certain non-linear characteristics and the introduction of MNLR improved the quality of prediction.

NEWLY DESIGNED COMPOUNDS

The main objective of the construction of QSAR models is the prediction of the activity of new candidate molecules that are more active than the studied molecules. This will be simple if we could understand the effect of each descriptor on the activity and the right choice of substitutes that vary these descriptors in the right direction.

According to the equation (1) and taking into account the sign of the values of the descriptors we could say that the activity increases with:

- The decrease in the refractive index (n)
- The increase in total energy (E_T)
- The decrease in chemical potential (μ)

The greater the absolute value of the t-test, the greater the influence of the descriptor. According to the obtained results (Table 7), the chemical potential μ has a stronger influence than the other two descriptors (n and E_T). The fact that the chemical potential is proportional to the energies of the HOMO and LUMO boundary orbitals ($\mu=(E_{HOMO}+E_{LUMO})/2$) allows us to conclude that a decrease of μ is equivalent to a decrease of the energy of the boundary orbitals, thus increasing the electrophile character of the molecule. Finally, to decrease the chemical potential, in other words, to increase the activity, it is necessary to introduce electron acceptor groups (deactivating groups such as NO_2, CN, CHO,...) which makes the molecule react as an electrophile.

Taking into account the above results, we introduced new substituents and then we calculated the activities of the proposed molecules. The structures of the proposed molecules and the pIC_{50} values theoretically predicted by

the MLR model as well as the corresponding levers are shown in the following table.

The proposed compounds **1p-4p** (Table10) showed higher activities than the most active compound 18 in the existing system (Table 1) and the corresponding lever values were found to be less than h*=0.522.

These results confirm that these compounds might be synthesized and evaluated as anti-CVB3 agents.

CONCLUSION

In this study, two QSAR models (MLR and MNLR) were constructed linking the inhibitory activity of a set of pleconaril-derived compounds (anti-CVB3) to three descriptors: refractive index (n), total energy (E_T) and chemical potential (μ). The set of the obtained results (statistical parameters, internal validation, external validation, and the field of applicability) showed that the two used models seem to be useful, although the advantage is to the non-linear model (MNLR). The strength of this study is the design of new candidate compounds that are more active than the studied compounds and that might be effective as antiviral agents against CVB3.

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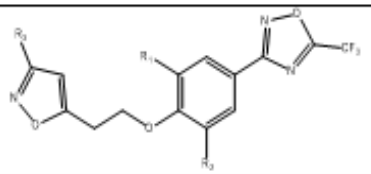
Table1: Chemical structures and corresponding activities



N°	R_1	R_2	R_3	pIC_{50}
1	Me	Me	MeOCO	7.222
2	Me	Me	EtOCO	7.155
3[b]	H	Me	EtOCO	7.208
4	H	MeO	EtOCO	6.310
5	H	NO_2	EtOCO	6.830
6	H	F	EtOCO	6.664
7	H	CF_3	EtOCO	7.523
8	MeO	NO_2	EtOCO	5.925
9	Me	Me	$(Me)_2CHOCO$	6.721
10	Me	Me	Ph	6.854
11[b]	Me	Me	NH_2CO	7.155
12	Me	Me	MeNHCO	7.222
13	Me	Me	EtNHCO	7.301
14	Me	Me	$PhCH_2NHCO$	5.857
15	Me	Me	Me_2NCO	7.699
16[b]	H	H	Me_2NCO	6.339
17	H	Me	Me_2NCO	7.523
18[a]	H	NO_2	Me_2NCO	8.523
19	H	F	Me_2NCO	7.602
20	H	CF_3	Me_2NCO	6.796
21[b]	MeO	NO_2	Me_2NCO	6.438
22	H	CF_3	MeNHCO	7.699
23	H	NH_2	EtOCO	4.699
24	H	Pyrrolyl	EtOCO	4.991
25[b]	H	$NCHNMe_2$	EtOCO	4.700
26	H	NH_2	$(Me)_2NCO$	5.350
27	H	NHCOMe	$(Me)_2NCO$	4.521
28	H	Pyrrolyl	$(Me)_2NCO$	6.086

((a): more active (b): test set)

Table 2: Software used to calculate the selected descriptors and models construction and validation

• Gaussian 09W	Highest Occupied Molecular Orbital Energy $E_{HOMO}(eV)$; Lowest Unoccupied Molecular Orbital Energy $E_{LUMO}(eV)$; Hardness η(eV)=((E$_{LUMO}$-E$_{HOMO}$))/2; Dipole moment μ_D (Debye) ; Chimical Potential μ(eV) =(E$_{LUMO}$+E$_{HOMO}$)/2;Electrophilicity Index ω(eV) =χ^2/2η; Total Energy E$_T$(eV); Energy Gap between $EHOMO$ and $ELUMO$ values $EGap(eV)$; Activation Enrgy Ea(eV) ; Wavelength of Absorption Maximum λ_{max} (nm) ; Oscillation force $f_{(so)}$.
• ChemSketchACD Labs	Molecular Weight MW (cm^3); Molar Refractivity MR (cm^3) ; Molecular Volume MV (cm^3) ; Parachor Pc (cm^3); Index of Refraction (n); Surface Tensionγ (dyne/cm); Density D (g/cm^3) ; Polarisabilty αe (cm^3) .
• MarvinSketch 20.2	Partition Coefficient log P ; Polar Surface Area PSA (A°)^2.
• Chemoffice 2016	Solubility logS ; Winner Index IW.
• XLSTAT 2016.02.284	Models construction and validation.
• MATLAB 2018a	

Table 3: Calculated values of the different descriptors

N°	IC$_{50}$	pIC$_{50}$	MW	MR	MV	Pc	n	γ	D	αe	logP	PSA	logS	IW	E$_{HOMO}$	E$_{LUMO}$	E$_T$	ΔE	μ_D	E$_a$	λ_{max}	f$_{(SO)}$	η	μ	ω
1	0,06	7,222	425,359	96,55	321,6	816,7	1,512	41,5	1,322	38,27	4,88	100,48	-5,5396	3015,00	-6,327	-1,662	-41780,131	4,665	3,217	4,433	279,680	0,0002	2,333	-3,995	3,420
2	0,07	7,155	439,385	101,18	338,1	856,5	1,510	41,1	1,299	40,11	5,23	100,48	-5,8805	3342,00	-6,576	-1,709	-43919,92	4,867	2,714	1,528	2098,150	0,0039	2,434	-4,143	3,526
3	0,062	7,208	425,359	96,36	321,8	818,9	1,510	41,8	1,321	38,20	4,73	100,48	-5,6817	3150,00	-6,2	-1,608	-42850,122	4,592	3,439	4,281	289,640	0,0003	2,296	-3,904	3,319
4	0,49	6,310	441,538	98,21	329,6	837,9	1,507	41,7	1,339	38,93	4,04	109,71	-5,3006	3368,00	-6,198	-1,652	-44896,296	4,546	3,743	1,469	843,760	0,0006	2,273	-3,925	3,389
5	0,148	6,830	470,356	102,47	334,6	876,6	1,524	47,0	1,405	40,62	4,12	143,62	-5,1920	3868,00	-7,287	-2,674	-47344,426	4,613	4,330	1,723	719,440	0,0004	2,307	-4,981	5,377
6	0,217	6,664	429,322	91,52	309,8	788,3	1,502	41,9	1,385	36,28	4,38	100,48	-5,7121	3150,00	-6,396	-1,789	-44480,186	4,607	3,340	1,558	795,720	0,0007	2,304	-4,093	3,635
7	0,03	7,523	479,330	96,51	339,1	838,4	1,481	37,3	1,413	38,26	5,11	100,48	-6,4989	3810,00	-6,689	-1,917	-50951,229	4,772	3,911	1,823	680,300	0,0001	2,386	-4,303	3,880
8	1,189	5,925	486,355	104,75	341,4	893,4	1,525	46,8	1,424	41,52	3,99	152,85	-5,3009	4025,00	-6,821	-2,541	-50460,765	4,28	4,867	1,608	771,030	0,0004	2,140	-4,681	5,120

N°	IC_{50}	pIC_{50}	MW	MR	MV	Pc	n	γ	D	αe	logP	PSA	logS	IW	E_{HOMO}	E_{LUMO}	E_T	ΔE	μ_D	E_a	λ_{max}	$f_{(SO)}$	η	μ	ω
9	0,19	6,721	453,412	105,77	355,0	893,7	1,507	40,1	1,277	41,93	5,64	100,48	-6,2521	3671,00	-6,573	-1,707	-44989,91	4,866	2,866	0,848	1462,810	0,0018	2,433	-4,140	3,522
10	0,14	6,854	443,418	109,37	349,0	885,2	1,539	41,3	1,270	43,35	6,88	74,18	-7,3703	3640,00	-6,473	-1,69	-42936,741	4,783	4,159	1,237	1002,290	0,0000	2,392	-4,082	3,483
11	0,07	7,155	410,347	93,70	302,4	786,3	1,531	45,6	1,356	37,14	4,00	117,27	-4,9466	2718,00	-6,588	-1,722	-41239,937	4,866	2,242	1,526	812,470	0,0005	2,433	-4,155	3,548
12	0,06	7,222	424,374	98,40	324,1	825,0	1,519	41,9	1,309	39,00	4,24	103,28	-5,2015	3015,00	-6,581	-1,716	-42309,679	4,865	2,414	1,494	829,650	0,0000	2,433	-4,149	3,538
13	0,05	7,301	438,400	103,03	340,6	864,8	1,516	41,5	1,287	40,84	4,59	103,28	-5,5346	3342,00	-6,578	-1,714	-43379,616	4,864	2,552	1,486	834,510	0,0000	2,432	-4,146	3,534
14	1,39	5,857	500,470	122,88	384,8	997,1	1,551	45,0	1,300	48,71	5,96	103,28	-7,0567	5274,00	-6,575	-1,719	-48597,094	4,856	2,213	1,355	915,160	0,0000	2,428	-4,147	3,542
15	0,02	7,699	438,400	103,16	339,0	862,5	1,520	41,8	1,293	40,89	4,47	94,49	-5,3490	3314,00	-6,562	-1,701	-43379,152	4,861	2,924	1,223	1014,140	0,0003	2,431	-4,132	3,511
16	0,458	6,339	410,347	93,51	306,4	787,2	1,522	43,5	1,338	37,07	3,47	94,49	-4,9510	2936,00	-6,314	-1,654	-41239,364	4,66	3,233	1,535	808,000	0,0007	2,330	-3,984	3,406
17	0,03	7,523	440,373	100,19	330,4	843,9	1,518	42,5	1,332	39,71	3,28	103,72	-4,7691	3340,00	-6,853	-1,615	-44355,61	5,238	2,711	1,408	880,370	0,0005	2,619	-4,234	3,422
18	0,003	8,523	455,345	100,05	318,3	842,7	1,541	49,1	1,430	39,66	3,43	137,63	-4,9564	3559,00	-6,656	-2,44	-46803,733	4,216	4,419	1,596	776,670	0,0006	2,108	-4,548	4,906
19	0,025	7,602	428,338	93,50	310,7	794,4	1,513	42,7	1,378	37,06	3,62	94,49	-5,1805	3123,00	-6,383	-1,781	-43939,42	4,602	2,977	1,439	861,570	0,0006	2,301	-4,082	3,621
20	0,16	6,796	478,345	98,49	340,0	844,4	1,491	38,0	1,406	39,04	4,35	94,49	-5,9674	3780,00	-6,65	-1,908	-50410,465	4,742	3,321	1,570	789,670	0,0006	2,371	-4,279	3,861
21	0,365	6,438	485,371	106,73	342,3	899,4	1,535	47,6	1,417	42,31	3,23	146,86	-4,7693	3994,00	-6,566	-2,402	-49919,902	4,164	5,242	1,521	815,010	0,0000	2,082	-4,484	4,829
22	0,02	7,699	464,319	93,73	325,0	806,9	1,488	37,9	1,428	37,15	4,12	103,28	-5,8205	3454,00	-6,681	-1,912	-49340,772	4,769	3,374	1,623	763,730	0,0000	2,385	-4,297	3,871
23	20,02	4,699	426,347	95,77	307,8	807,0	1,534	47,2	1,384	37,96	3,45	126,50	-5,1453	3150,00	-5,532	-1,523	-43286,52	4,009	3,807	1,517	817,280	0,0005	2,005	-3,528	3,104

N°	IC_{50}	pIC_{50}	MW	MR	MV	Pc	n	γ	D	αe	logP	PSA	logS	IW	E_{HOMO}	E_{LUMO}	E_T	ΔE	μ_D	E_a	λ_{max}	$f_{(SO)}$	η	μ	ω
24	10,22	4,991	476,405	113,22	333,6	868,2	1,594	45,8	1,420	44,88	5,14	105,41	-6,8028	4091,00	-5,911	-1,927	-47466,405	3,984	2,383	1,069	1159,660	0,002	1,992	-3,919	3,855
25	19,95	4, 700	481,425	113,85	349,0	889,1	1,565	42,0	1,370	45,13	4,08	116,08	-5,7915	4181,00	-5,653	-1,538	-47968,862	4,115	4,914	1,291	960,690	0,0009	2,058	-3,596	3,142
26	4,47	5,350	425,362	97,74	308,7	813,1	1,545	48,0	1,377	38,75	2,69	120,51	-4,6137	3123,00	-5,521	-1,515	-42745,752	4,006	4,448	1,111	1116,150	0,0000	2,003	-3,518	3,089
27	30,14	4,521	467,399	107,78	339,5	891,0	1,647	47,3	1,376	42,72	2,80	123,59	-4,8301	3838,00	-6,072	-1,817	-46899,782	4,255	3,225	1,045	1186,740	0,0002	2,128	-3,945	3,657
28	0,82	6,086	475,421	115,61	337,0	875,6	1,601	45,5	1,410	45,83	4,39	99,42	-6,2713	4060,00	-6,662	-1,782	-46925,713	4,88	3,200	1,063	1166,800	0,0020	2,440	-4,222	3,6527

Table 4: Matrix of correlation

	pIC_{50}	MW	MR	MV	Pc	n	γ	D	αe	logP	PSA	logS	IW	E_{HOMO}	E_{LUMO}	E_T	ΔE	μ_D	Ea	λmax	$f_{(SO)}$	η	μ	ω
pIC_{50}	1																							
MW	-0,276	1																						
MR	**-0,451**	**0,700**	1																					
MV	-0,156	**0,751**	**0,816**	1																				
Pc	-0,299	**0,769**	**0,905**	**0,940**	1																			
n	**-0,657**	0,287	**0,629**	0,180	**0,397**	1																		
γ	**-0,410**	0,069	0,259	-0,16	0,182	**0,622**	1																	
D	-0,168	0,429	-0,10	-0,27	-0,163	0,160	0,334	1																
αe	-0,451	0,700	1,000	0,816	0,905	0,629	0,259	-0,10	1															
logP	0,175	0,182	0,378	0,564	0,395	-0,19	-0,48	-0,50	0,378	1														
PSA	-0,252	0,323	0,056	-0,06	0,176	0,216	0,709	0,565	0,056	-0,56	1													
logS	0,052	-0,41	-0,52	-0,60	-0,457	-0,012	0,427	0,219	-0,524	-0,90	0,540	1												
IW	-0,368	0,922	0,857	0,849	0,897	0,387	0,150	0,185	0,857	0,322	0,195	-0,53	1											
E_{HOMO}	-0,666	-0,23	0,014	-0,26	-0,194	0,356	0,199	-0,00	0,014	-0,23	-0,05	0,080	-0,13	1										
E_{LUMO}	-0,192	**-0,508**	-0,08	-0,12	-0,253	0,005	-0,38	-0,58	-0,086	0,143	-0,69	-0,13	-0,32	0,585	1									
E_T	0,146	**-0,927**	**-0,39**	**-0,526**	**-0,508**	-0,073	0,033	**-0,633**	**-0,391**	-0,01	**-0,380**	0,268	**-0,743**	0,272	**0,584**	1								
ΔE	**0,624**	-0,184	-0,09	0,200	-0,001	**-0,423**	**-0,592**	**-0,529**	-0,09	**0,411**	**-0,575**	-0,22	-0,13	**-0,662**	0,222	0,214	1							
μ_D	-0,193	0,314	0,013	-0,01	0,082	0,005	0,298	**0,472**	0,013	-0,29	**0,575**	0,219	0,162	0,126	**-0,471**	**-0,400**	**-0,587**	1						
Ea	0,285	-0,27	-0,33	-0,22	-0,280	-0,316	-0,187	-0,10	-0,33	0,107	-0,06	0,056	-0,29	-0,007	0,059	0,232	0,063	0,027	1					
λ_{max}	-0,214	0,109	0,334	0,276	0,283	0,276	0,032	-0,21	0,334	0,156	-0,11	-0,17	0,163	0,066	0,146	0,004	0,056	-0,21	**0,630**	1				
$f_{(SO)}$	-0,091	0,034	0,183	0,071	0,052	0,132	-0,05	-0,06	0,183	0,228	-0,14	-0,23	0,047	0,011	0,088	0,033	0,068	-0,25	-0,19	**0,760**	1			
η	**0,624**	-0,184	-0,09	0,200	-0,001	**-0,423**	**-0,592**	**-0,529**	-0,09	**0,411**	**-0,575**	-0,22	-0,13	**-0,662**	0,222	0,214	**1,000**	**-0,587**	0,063	0,056	0,068	1		
μ	**-0,516**	**0,398**	-0,03	-0,22	0,246	0,228	-0,06	-0,287	-0,033	-0,07	-0,37	-0,01	-0,24	**0,919**	**0,858**	**0,457**	-0,311	-0,15	0,024	0,113	0,050	-0,311	1	
ω	0,222	**0,496**	0,086	0,134	0,260	-0,024	0,372	**0,551**	0,086	-0,13	**0,683**	0,143	0,319	**-0,619**	**-0,998**	**-0,568**	-0,179	**0,456**	0,053	-0,14	-0,087	-0,179	**-0,879**	1

Table 5: Multicollinearity statistics

	n	E_T	μ
Tolerance	0.906	0.739	0.688
VIF	1.104	1.353	1.454

Table 6: Caracteristics of the MLR model parameters

Source	Value	Standard Error	t	Pr > $\|t\|$
Constant	16.8258	3.845	4.376	0.000
n	-11.4683	2.126	-5.394	< 0.001
E_T	0.0002	0.000	5.349	< 0.001
μ	-3.7219	0.406	-9.156	< 0.001

Table 7: Comparison of model parameters (MLR) with Golbraikh and Tropsha criteria

	Parameter	Model score	Threshold
Fitting criteria	$R^2 = 1 - \frac{\sum(Y_{obs} - Y_{calc})^2}{\sum(Y_{obs} - \overline{Y}_{obs})^2}$	0.893	> 0.6
	$R^2_{adj} = \frac{(N-1).R^2 - k}{N-k-1}$	0.876	> 0.6
	$MSE = \frac{\sum(Y_{obs} - Y_{calc})^2}{N}$	0.135	A low value
	$RMSE = \sqrt{\frac{\sum(Y_{obs} - Y_{calc})^2}{N}}$	0.367	A low value
	$F = \frac{\sum(Y_{calc} - \overline{Y}_{calc})^2.N - k - 1}{\sum(Y_{obs} - Y_{calc})^2.k}$	52.972	A high value
Internal validation	$Q^2_{CV} = 1 - \frac{\sum(Y_{calc} - Y_{obs})^2}{\sum(Y_{obs} - \overline{Y}_{obs})^2}$	0.837	> 0.5
	R_{Rand} (Average of the 100 $R_{Rand(i)}$)	0.149	$< R$
	R^2_{Rand} (Average of the 100 $R^2_{Rand(i)}$)	0.360	$< R^2$
	$Q^2_{CV\,LMO(Rand)}$ (Average of the 100 $Q^2_{CV\,LMO(Rand)(i)}$)	-0.254	$< Q^2_{CV}$
	$cR^2_p = R.\sqrt{(R^2 - R^2_{Rand})}$	0.828	> 0.5
	$\bar{r}^2_{m(CV\,LMO)} = \frac{\left\|r^2_m + r'^2_m\right\|}{2}$	0.774	> 0.5
	$\Delta r^2_{m(CV\,LMO)} = \left\|r^2_m - r'^2_m\right\|$	0.066	< 0.2
External validation	$R^2_{test} = 1 - \frac{\sum(Y_{calc(test)} - Y_{obs(test)})^2}{\sum(Y_{obs(test)} - \overline{Y}_{obs(train)})^2}$	0.778	> 0.5

	Parameter	Model score	Threshold
Fitting criteria	$R^2 = 1 - \frac{\sum(Y_{obs} - Y_{calc})^2}{\sum(Y_{obs} - \overline{Y}_{obs})^2}$	0.893	> 0.6
	$R^2_{adj} = \frac{(N-1).R^2 - k}{N-k-1}$	0.876	> 0.6
	$MSE = \frac{\sum(Y_{obs} - Y_{calc})^2}{N}$	0.135	A low value
	$RMSE = \sqrt{\frac{\sum(Y_{obs} - Y_{calc})^2}{N}}$	0.367	A low value
	$F = \frac{\sum(Y_{calc} - \overline{Y}_{calc})^2.N - k - 1}{\sum(Y_{obs} - Y_{calc})^2.k}$	52.972	A high value
	$\overline{r}^2_{m(\text{test})} = \frac{\left\|r^2_m + r'^2_m\right\|}{2}$	0.594	> 0.5
	$\Delta r^2_{m(\text{test})} = \left\|r^2_m - r'^2_m\right\|$	0.193	< 0.2
	$\Delta r^2_{0(\text{test})} = \left\|r^2_0 - r'^2_0\right\|$	0.239	< 0.3
	$\frac{(r^2 - r^2_0)}{r^2}$	0.345	< 0.1
	$\frac{(r^2 - r'^2_0)}{r^2}$	0.038	< 0.1
	$K = \frac{\sum(Y_{obs}.Y_{calc})^2}{\sum(Y_{calc})^2}$	0.963	$0.85 \leq K \leq 1.15$
	$K' = \frac{\sum(Y_{obs}.Y_{calc})^2}{\sum(Y_{obs})^2}$	1.028	$0.85 \leq K' \leq 1.15$

Table 8: Statistical parameters of the MNLR model

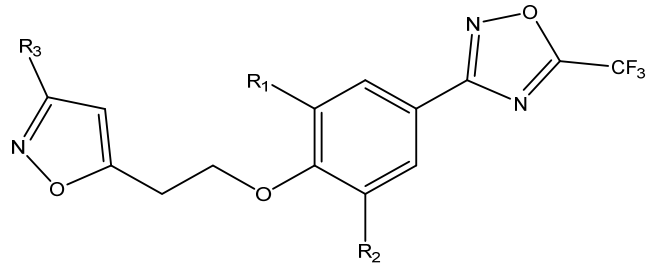
Model parameters							Internal and external validation							
N	R	R^2	R^2_{adj}	MSE	RMSE	F	$Q^2_{CV\,(LOO)}$	$Q^2_{CV\,LMO\,(Rand)}$	R_{Rand}	R^2_{Rand}	cR^2_p	R^2_{test}	K	K'
23	0.958	0.918	0.905	0.122	0.349	67.511	0.784	0.397	0.397	0.176	0.836	0.737	0.875	1.097

Table 9: Comparison of the observed values with those calculated by MLR and MNLR

N°	$pIC_{50(observed)}$	$pIC_{50(predicted)}$		N°	$pIC_{50(observed)}$	$pIC_{50(predicted)}$	
		MLR	MNLR			MLR	MNLR
1	7.222	7.137	7.166	**15**	7.699	7.279	7.220
2	7.155	7.341	7.292	**16**[b]	6.339	7.075	7.117
3[b]	7.208	6.636	6.581	**17**	7.523	7.513	7.501
4	6.310	6.395	6.221	**18**[a]	8.523	7.995	8.423
5	6.830	7.173	7.119	**19**	7.602	7.076	6.970
6	6.664	7.150	7.069	**20**	6.796	6.944	7.013
7	7.523	7.054	7.240	**21**[b]	6.438	7.287	7.551
8	5.925	6.437	6.314	**22**	7.699	7.230	7.330
9	6.721	7.179	7.082	**23**	4.699	4.886	5.085
10	6.854	6.951	6.849	**24**	4.991	4.931	4.681
11[b]	7.155	7.608	7.719	**25**[b]	4.700	3.975	3.873
12	7.222	7.538	7.579	**26**	5.350	4.816	5.061
13	7.301	7.377	7.343	**27**	4.521	4.518	4.689
14	5.857	6.078	5.794	**28**	6.086	6.072	6.033

((a): more active (b): test set)

Table 10: Values of descriptors, calculated anti- CVB3 activity pIC_{50} and leverages (h) for the new compounds



N°	R_1	R_2	R_3	n	E_T (eV)	μ (eV)	pIC_{50}	h
18	H	NO_2	$(Me)_2NCO$	1.541	-46803.733	-4.548	8.523	0.2260
1p	NO_2	NO_2	$(Me)_2NCO$	1.558	-52367.980	-5.033	8.644	0.2103
2p	NO_2	NO_2	NO_2	1.575	-51202.249	-5.637	10.898	0.4300
3p	CN	CN	CN	1.578	-42038.746	-5.265	11.063	0.4398
4p	CHO	CHO	CHO	1.581	-43759.955	-4.956	9.580	0.1939

دراسة مشتقات جديدة للبْلِكُونَارِيلْ (مركبات ايزوجزازول) المضاد للفيروسات كُوكْسَاكِي ب 3 باعتماد المقاربة "العلاقة الكمية نشاط – بنية ثنائية البعد"

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ملخص

نظرا لأعراضها الحادة، أصبح البحث عن دواء مضاد للفِيرُوسَات كُوكْسَاكِي ب 3 (CVB3) ضرورة ملحة ومستعجلة. 28 مركب مشتقة من البْلِكُونَارِيلْ (مضاد CVB3) كانت موضوع الدراسة باعتماد المقاربة " العلاقة الكمية نشاط – بنية ثنائية البعد" من أجل بناء نموذج يمكننا من التنبؤ بمركبات جديدة أكثر نشاطا من المركبات المدروسة. بتوظيف الانحدار الخطي المتعدد والانحدار غير الخطي المتعدد تم الحصول على نموذجين بنتائج جيدة. استثمار هذين النموذجين مكننا من اقتراح مركبات نشاطها أكبر مقارنة مع المركبات المدروسة. تعتبر هذه النتائج مرجعا مهما لأعمال تجريبية مقبلة.

الكلمات الدالة: العلاقة الكمية نشاط – بنية ثنائية البعد، الانحدار الخطي المتعدد، الانحدار غير الخطي المتعدد، فيروس كُوكْسَاكِي ب 3، البْلِكُونَارِيلْ، إيزُوكْسَازُولْ، مضاد فيروسات.

تاريخ استلام البحث 2020/5/29 وتاريخ قبوله للنشر 2021/4/17.

Theoretical studies of plant-based peptides targeting human angiotensin converting enzyme-related carboxypeptidase

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ABSTRACT

Background

The vulnerability of the lungs, intestine, heart and kidney to SARS-CoV-2 invasion is dependent on the high expression of angiotensin converting enzyme-related carboxypeptidase (ACE2) on the outer surface of the cells in these organs. This clear mode of interaction between SARS-CoV-2 spike proteins and ACE2 emphasizes the importance of ACE2 receptors in the spread of coronaviruses. This study investigated the binding potentials of some selected plant-based peptides (circulin A, kalata B1, Varv peptide E, palicourein, Vhl-1, griffithsin, cycloviolacin VY1) to ACE2 as a predictive approach in preventing SARS-CoV-2 invasion.

Methods

The peptides were retrieved from the antimicrobial peptide database and their respective physicochemical properties were predicted using ProtParam Tool. The binding mode and the binding free energies were computed through HawkDock servers while the structural flexibility and stability of the ACE2-peptide complexes were evaluated via the CABS-flex 2.0 server.

Results

It was observed that the binding scores for the peptides towards ACE2 showed good binding affinities with griffithsin having the best binding score through the Hawkdock rank while kalata B1 had the lowest binding score. The Molecular Mechanics/Generalized Born Surface Area analysis showed that the binding free energy ranges -39.99 and -3.96 kcal/mol with Vhl-1 having the highest free energy and palicourein having the least free energy.

Conclusions

The results of the study suggest that the selected plant-based peptides especially kalata B1, vhl-1, and cycloviolacin VY1 could be promising modulators of ACE2 and prevent the binding of the S1 domain of the SARS-CoV-2 S protein and consequent cellular entry of SARS-CoV-2.

Keywords: Peptides, ACE2, Binding affinity, Modulatory potential, SARS-COV.

INTRODUCTION

More than a decade ago, a novel coronavirus that infects humans, bats and certain other mammals, termed Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), caused an epidemic with ~ 10% case fatality, creating global panic and economic damage (Lu *et al*., 2014). Recently, another strain of the virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), caused an infectious disease (COVID-19) in human which was for the first time detected in Wuhan, China (Zhang *et al*., 2020). Since then, person to person transmission of the

Received on 9/6/2020 and Accepted for Publication on 17/4/2021.

infectious disease has been on the increase, causing a global pandemic with over 3,946,000 confirmed cases and more than 271,600 deaths (ECDC, 2020). Presently, there is no specific therapies available for the treatment of COVID-19. Social distancing, patient isolation and supportive medical care make up the current management for this infectious disease (Zhang *et al*., 2020).

Coronaviruses use spike proteins (type 1 transmembrane glycoproteins) to mediate viral infection; the subunits of the spike proteins are used to achieve viral fusion and entry (Xia *et al*., 2019). These spike proteins are made up of two subunits, S1 and S2, coronaviruses bind to cellular receptor through S1 subunit's receptor binding domain (RBD), this results in conformational changes of the S2 subunit that leads to the insertion of fusion peptide into the host cell membrane (Xia *et al*., 2019). The amino acid sequencing of the original SARS-CoV and SARS-CoV-2 spike proteins showed that they share 76.5% identity, also, computer modeling of these spiked proteins revealed identical 3D RBD structures (Xu *et al*., 2020). Similar to SARS-CoV and other coronaviruses, SARS-CoV-2 is able to use human angiotensin converting enzyme-related carboxypeptidase (hACE2) as a receptor to infect human cells (Wu *et al*., 2012; Xia *et al*., 2020). Reports from analysis showed that glutamine (residue 394) of SARS-CoV-2 RBD is recognized by lysine (residue 31) on hACE2 receptor, further analysis revealed that SARS-CoV-2 spike proteins recognizes hACE2 more efficiently than SARS-CoV, this is suggested as the reason for the increase in person to person transmission (Wan *et al*., 2020; Zhang *et al*., 2020).

ACE2 is a type 1 integral membrane glycoprotein that is expressed and attached to the outer surface of cells in the lungs, heart, kidney and intestine (Yan *et al*., 2020). ACE2 plays important role in maintaining the renin-angiotensin-aldosterone system (RAAS) balance by catalyzing the cleavage of angiotensin II (vasoconstrictor peptide) into angiotensin 1-7 (vasodilator peptides) (Yan *et al*., 2020). Alveolar epithelial type II cells have been reported to have a high expression of ACE2, about 83%, this suggests that these cells are potential reservoirs for viral invasion making the lung the most vulnerable organ target (Zhang *et al*., 2020). Hashimoto *et al*. (2012) also reported high expression of ACE2 receptors on the luminal surface of intestinal epithelial cells, where they function additionally as co-receptors for amino acid resorption from food. Zhang *et al*. (2020) proposed that the intestine might also be a major entry site for SARS-CoV-2 and that eating food from Wuhan market might have initiated the outbreak of COVID-19 in China. Furthermore, the broad tissue distribution of ACE2 in organs explains the multi-organ dysfunction observed in patients with severe forms of COVID-19 (Huang *et al*., 2020). It has also been speculated that the severity of the infectious disease in the elderly, especially those with cardiovascular comorbidities is influenced by the use of RAAS blockers that increase the expression of ACE2 (Zheng *et al*., 2020). This has led to the hypothesis that decreasing the levels of ACE2 in cells might help in fighting the spread of COVID-19, however ACE2 also play major role in protecting the lung from virus induced injury by increasing the production of angiotensin 1-7 (Tikellis and Thomas, 2012). Also, it is worthy of note that the binding of spike proteins to ACE2 (especially in the lungs) results in the reduced expression and enzymatic activity of ACE2, due to enhanced internalization and may contribute to lung damage that is observed in severe cases of COVID-19 (Jia, 2016).

In a quest for an effective treatment for COVID-19, several potential therapeutic approaches have been proposed such as inhibition of heptad repeat 1 (HR1) in the S2 subunit, inhibition of transmembrane protease serine 2 activity, inhibition of viral six-helical bundle (6-HB) formation, blocking ACE2 receptor, delivering excessive soluble form of ACE2 and spike protein-based vaccine (Yu *et al*., 2016; Zhang *et al*., 2020). The mode of interaction between SARS-CoV-2 spike proteins RBD and ACE2 emphasizes the importance of ACE2 receptors in the spread of COVID-19. Several antiviral drugs have been

proposed as repurposing drugs to impair the binding and replication of the virus; however, these agents might not be healthy alternatives as besides being expensive they produce a wide spectrum of adverse effects. Therefore, this study attempts to investigate the binding potentials of selected plant-based peptides to ACE2

Materials and Methods

Ab Initio modelling of the selected peptides

The 3D structures of the selected peptides were obtained from the Protein Data Bank (https://www.rcsb.org/). The 3D structures of cycloviolacin VY1 and varv peptide E were predicted by submitting their respective FASTA amino acid sequence into I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), an automated modelling server (Yang *et al*., 2015; Zhang *et al*., 2017). The best model for each peptide was selected based on the C-scores and was further validated using the Structure Analysis and Verification server SAVES v5.0 (https://servicesn.mbi.ucla.edu/SAVES/) (Pontius *et al*., 1996).

Physicochemical characterization

The physicochemical characterization of the respective **peptides** was predicted using ProtParam Tool on the SIB ExPASy webserver ProtParam tool (https://www.expasy.org/tools/) using mammalian as the defined organism (Artimo *et al*., 2012). The computed parameters include the molecular weight, theoretical pI, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity.

Database screening

The database screening of peptides with anti-viral properties was conducted using APD3 database (http://aps.unmc.edu/AP/main.php) (Wang *et al*., 2016). The selections were based on the reported biological activities a presented in the APD3 database.

Molecular docking

The structure of native human angiotensin converting enzyme-related carboxypeptidase (ACE2) was retrieved from the Protein Data Bank (https://www.rcsb.org/) with the PDB ID: IR42. The structural bioinformatics studies of the selected peptides with the human ACE2 were computed using the HawkDock server (http://cadd.zju.edu.cn/hawkdock/) integrated with the ATTRACT docking algorithm, the HawkRank scoring function and the Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) free energy decomposition analysis (Feng *et al*., 2017; Weng *et al*., 2019) and the interactions were visualized using PyMOL ver. 1.1eval (De Lano Scientific LLC, CA, USA). All the peptides and the protein were converted into Auto Dock Pdbqt format. The binding energy between the peptides and the protein were computed.

Binding Mode of Docked Complexes

Interactions within the docked complexes were investigated through the Protein Interactions Calculator webserver (http://pic.mbu.iisc.ernet.in/) using the docked complexes (Tina *et al*., 2007). The hydrophobic Interactions, disulphide bridges, hydrogen bonds and ionic Interactions were determined.

Molecular Dynamics (MD) Simulations

The molecular dynamics simulations were carried out using the CABS-flex 2.0 server to evaluate the structural flexibility and stability within a nanosecond time scale of the ACE2-peptide complexes (Kuriata *et al*., 2018). The root-mean-square fluctuations and contact maps were obtained.

Results and Discussion

Ab Initio modelling of the selected peptides

The design of new antiviral molecules is a worldwide priority, especially for the current pandemic viral disease known as COVID-19 due to no specific vaccine or

treatment. The present study employed computational study, as a predictive approach in the drug design. The theoretical models employed for the selection of effective receptor-binding ligands for ACE2 resulted in seven short-listed plant-based peptides belonging to different families of plant species. Natural products of plants origin are considered good chemopreventive agents because of their low toxicities and potential efficacies (Crowell, 2005). Moreover, peptides had been identified to show diverse therapeutic potentials such as antiviral, anticancer, antimicrobial, antiparasitic, etc (Mehta *et al*., 2014; Mustafa *et al*., 2019). Peptides have been identified as effective receptor-binding ligand (Roxin and Zheng, 2012). The selected peptides AP3 ID, peptide names, PDB ID, FASTA sequence, length code, and the corresponding natural sources are presented in Table 1.

Table 1: Identifications of the selected compounds

AP3 ID	Name	PDB ID	FASTA sequence	Length	Source
AP00274	Circulin A	1BH4	GIPCGESCVWIPCISAAL GCSCKNKVCYRN	30	*Chassalia parviflora*
AP00729	Kalata B1	1K48	GLPVCGETCVGGTCNTP GCTCSWPVCTRN	29	*Oldenlandia affinis*
AP01030	Varv peptide E	N/A	GLPICGETCVGGTCNTP GCSCSWPVCTRN	31	*Viola arvensis*
AP01034	Palicourein	1R1F	GDPTFCGETCRVIPVCTY SAALGCTCDDRSDGLCK RN	37	*Palicourea condensata*
AP01058	Vhl-1	1ZA8	SISCGESCAMISFCFTEVI GCSCKNKVCYLN	31	*Viola hederaceae*
AP02133	Griffithsin	2GTY	SLTHRKFGGSGGSPFSGL SSIAVRSGSYLDAIIIDGV HHGGSGGNLSPTFTFGS GEYISNMTIRSGDYIDNI SFETNMGRRFGPYGGSG	121	*Griffithsia* sp
AP02571	Cycloviolacin VY1	N/A	GSANTLSNVKVIQINGSA GDYLDSLDIYYEQY CGESCVFIPCITTVLGCS CSIKVCYKNGSIP	31	*Viola yedoensis*

N/A=Not available

Physicochemical characterization

The result of the predicted physicochemical characterization (Table 2) shows that palicourein, circulin A and kalata B1 has higher and better half-life (30 h) than either Vhl-1 and Griffithsin (1.9 h) or cycloviolacin VY1 and Varv peptide E (1.2 h) that may be due to amino acid composition and cyclic property of the peptides (Vlieghe *et al*., 2010) suggesting that palicourein, circulin A and kalata B1 could resist the proteases degradation and express high bioavailability. The result is in agreement with Mathur *et al*. (2018) who reported that susceptible enzymatic degradation of peptides due to short half-life

reduces their bioavailability and blocked their therapeutic development despite their effective potency over small drugs. However, Vhl-1 and cycloviolacin VY1 bioavailability could be improved either by D-amino acid incorporation (Vlieghe *et al*., 2010) or α-aminoxy amino acid incorporation (Chen *et al*., 2011). The instability index of the peptides was evaluated and the result indicates that Varv peptide E, circulin A, cycloviolacin VY1 and griffithsin have a better instability value (<40) while palicourein and Vhl-1 high instability value and kalata B1 could be slightly stable with instability value of 46.59. Also, the predictive assessment of aliphatic index reveal that varv peptide E, circulin A, Vhl-1, cycloviolacin VY1 and griffithsin have high aliphatic index suggesting the presence of high portion of aliphatic residues such as Leu, Ile and Val which could be responsible for the antiviral potential of the peptides as reported by Chang and Yang (2013). Moreover, the aliphatic index correlates with the instability index except Vhl-1 with moderate high instability index.

Furthermore, the negative GRAVY score result of palicourein and griffithsin indicates their hydrophilic potency suggesting high absorption, ease metabolism and less toxicity but their membrane permeability is slightly poor. However, varv peptide E, circulin A, vhl-1 and cycloviolacin VY1 exhibit better lipophilicity potential due to positive GRAVY score suggesting their ease membrane permeation with difficult solubility and metabolism. This implies that varv peptide E, circulin A, vhl-1 and cycloviolacin VY1 are likely to be toxic thus the intake concentration should be reduced. Interestingly the GRAVY score for kalata B1 is close to zero indicating a balance between the hydrophilic and lipophilic potential of the peptide which suggests a better absorption, metabolism, permeability but less toxic.

Table 2: Physicochemical characterization of the selected peptides

Name	Mol wt (Da)	Theoretical pI	Half-life (h)	Instability index	Aliphatic index	*GRAVY
Circulin A	3175.78	8.33	30	26.56	78.00	0.417
Kalata B1	2916.34	5.96	30	46.59	43.45	0.152
Varv peptide E	3225.88	7.77	1.2	17.44	90.97	0.874
Palicourein	3928.43	4.78	30	60.26	52.70	-0.189
Vhl-1	3340.94	5.85	1.9	56.12	72.26	0.690
Griffithsin	12690.85	5.39	1.9	39.86	70.91	-0.240
Cycloviolacin VY1	3225.88	7.77	1.2	17.44	90.97	0.874

*GRAVY= Grand average of hydropathicity

Binding energy

The Hawkdock server generated 10 predictive models for each of the protein-peptide complex with distinct score values. The first model of each interaction was selected for *in silico* characterization being least value which suggests a model with a high confidence was selected as a final model. The binding scores of the selected peptides after docking with ACE2 are listed in Table 3. The result of the comparative analysis reveals that the binding affinity of the peptides decreased in this order: griffithsin>palicourein>cycloviolacin VY1>vhl-1>circulin A>varv peptide E>kalata B1 with respective binding scores -5314.19>-4215.77>-3987.52>-3690.70>-3637.79>-3585.30>-3280.71. However, studies have shown that docking scores are not satisfactorily presenting the accurate protein-ligand binding affinity (Suenaga *et al*., 2012). Further analysis was performed to predict the free binding energies of the protein–peptide docked

complexes through the MM/GBSA analysis. The MM/GBSA predicted free energies reveals that Vhl-1 had the highest binding free energy (-39.99 kcal/mol) followed by Cycloviolacin VY1 (-38.31 kcal/mol) and kalata B1(-37.49 kcal/mol). Palicourein had the lowest bind free energy followed by griffithsin with values of -3.96 and -19.74 kcal/mol, respectively. The scatter plot obtained from the values of binding free energies of the peptides using the MM/GBSA rescoring against the binding affinity derived from the molecular docking indicates significant discrepancy with low correlation (Figure 1). Thus, the binding affinity could not be efficient for ranking the binding energies as indicated by a small correlation coefficient. The illustrations of the interaction of ACE2 with peptides are shown in Figure 2 along with their corresponding interacting residues identified through the Protein Interactions Calculator server. Three noticeable binding sites were identified in the ACE2 structure. Circulin A, kalata B1, varv peptide E, vhl-1, and cycloviolacin VY1 were revealed to occupy the major binding site on ACE2 while palicourein and griffithsin were bound at different pockets.

Table 3: Binding scores and the MM-GBSA free binding energy

Name	HawkRank Scores	MM/GBSA free energy (kcal/mol)
Circulin A	-3637.79	-26.33
Kalata B1	-3280.71	-37.49
Varv peptide E	-3585.30	-31.26
Palicourein	-4215.77	-3.96
Vhl-1	-3690.70	-39.99
Griffithsin	-5314.19	-19.74
Cycloviolacin VY1	-3987.52	-38.31

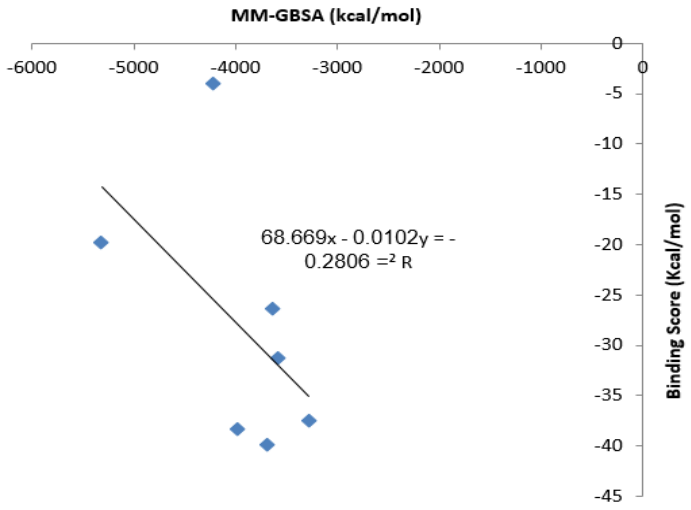

Figure 1: The scatter plot of MM/GBSA binding free energy versus binding affinity.

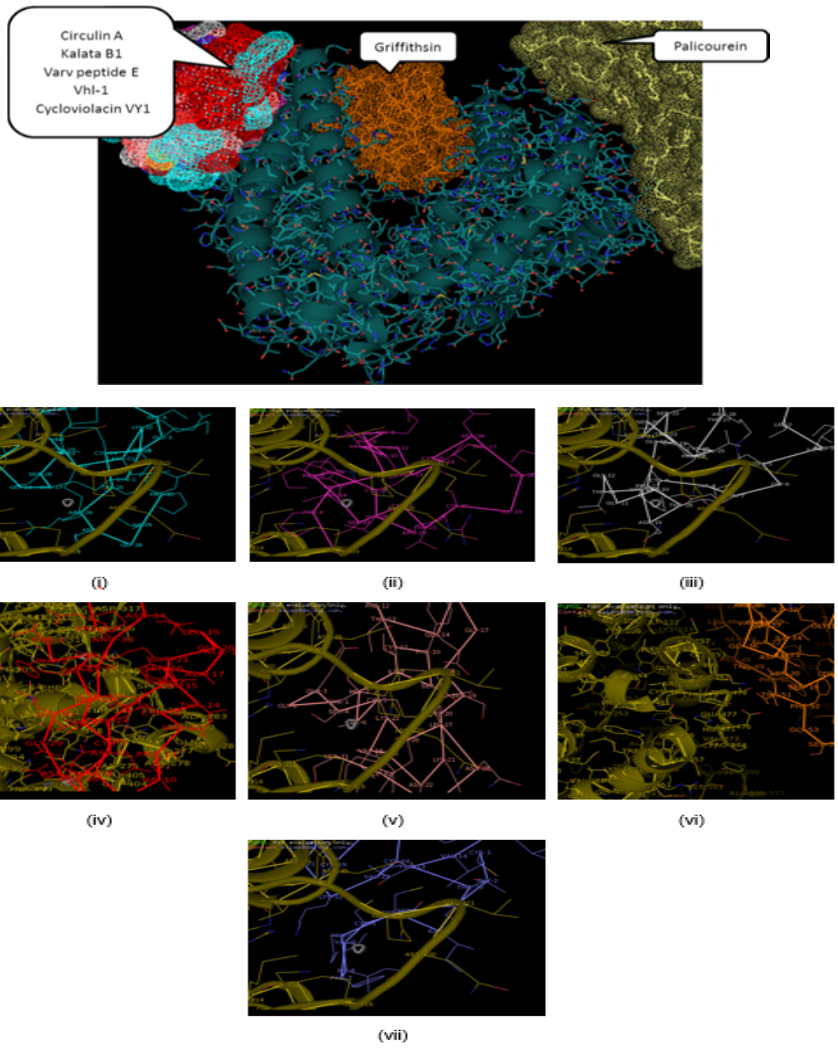

Figure 2: 3D illustrations of (A) the binding positions and (B) interacting residues of complex of ACE2 with (i) Circulin A, (ii) Kalata B1, (iii) Varv peptide E, (iv) Palicourein, (v) Vhl-1, (vi) Griffithsin, (vii) Cycloviolacin VY1

ACE2-peptide interactions

The interactions of the ACE2 with the peptides are presented in Tables 4 and 5. The main hydrophobic contacts within 5 Angstroms were established through ALA-28, MET-314, and PRO-318 in ACE2 and PHE-12, and ILE-30 in Vhl-1. TRP-30, TYR-23, and LEU-27 located in the main binding pocket of ACE2 contributed to the hydrophobic interactions involving PRO-9 and ILE-21 of cycloviolacin VY1 (Table 4). In addition, the hydrophobic interactions were established through PRO-271 and VAL-346 of ACE2 and VAL-9 and TYR-15 in palicourein while they occurred between ACE2 and griffithsin through VAL-154, PRO-117, TRP-145, and PRO-120, and LEU-46, PRO-48, and TYR-85, respectively. The predicted interaction also showed that TYR-23, LEU-27, TRP-30, and MET-314 from the ACE2 could be considered key interactions residues due to their frequent occurrences. There was no protein-protein disulphide bridges found in the interactions of the peptides with ACE2. However, ionic interaction within 6 A˚ were established in palicourein, griffithsin, and cycloviolacin

VY1with ACE2 while aromatic-aromatic interaction within 4.5 and 7 A˚ were found in theACE2 interactions with kalata B1 and vhl-1. Interactions through hydrogen bonding were found to exist between ACE2 and the peptides. These parameters demonstrated the binding strength between ACE2 and the peptides.

Table 4: Possible residues involving in the interactions of ACE2 and the peptides

Peptides	Hydrophobic Interactions*		Ionic Interactions#		Aromatic-Aromatic Interactions§	
	AR	PR	AR	PR	AR	PR
Circulin A	ALA-28	PRO-9				
	ALA-47	PRO-9				
	TYR-23	ALA-14				
	LEU-27	ALA-14				
	TYR-23	LEU-15				
Kalata B1	MET-314	LEU-3			TYR-23	TRP-24
	PRO-318	LEU-3				
	TRP-30	PRO-4				
	MET-314	PRO-4				
	MET-314	VAL-5				
	PRO-318	VAL-5				
	TYR-23	TRP-24				
	TYR-23	PRO-25				
	LEU-27	PRO-25				
	LEU-33	PRO-25				
	LEU-27	VAL-26				
	TRP-30	VAL-26				
Varv peptide E	MET-314	PRO-17			TYR-23	TRP-23
	TYR-23	TRP-23				
	LEU-27	TRP-23				
Palicourein	PRO-271	VAL-9	ASP-274	ARG-8		
	VAL-346	TYR-15	ASP-349	ARG-8		
			LYS-423	ASP-28		
			GLU-132	LYS-32		
			GLU-127	ARG-33		
Vhl-1	ALA-28	PHE-12				
	MET-314	ILE-30				
	PRO-318	ILE-30				
Griffithsin	VAL-154	LEU-46	ASP-453	ARG-145		
	PRO-117	PRO-48	ASP453	HIS-159		
	TRP-145	PRO-48				
	PRO-117	TYR-85				
	PRO-120	TYR-85				
Cycloviolacin VY1	TRP-30	PRO-9	ASP-337	LYS-22		
	TYR-23	ILE-21	GLU-39	LYS-26		
	LEU-27	ILE-21				

Interactions: * within 5 A˚; # within 6 A˚; § within 4.5 and 7 A˚. AR= ACE2 residues, PR=Peptide residues

Table 5: ACE2-peptide interactions involving hydrogen bonds

	Main Chain-Main Chain Hydrogen Bonds		Main Chain-Side Chain Hydrogen Bonds		Side Chain-Side Chain Hydrogen Bonds	
	AR	PR	AR	PR	AR	PR
Circulin A			TYR-23	LEU-15	ASP-337	SER-18
			GLU-311	ASN-27	ASN-31	CYS-19
			TYR-23	ALA-14	GLU-311	ASN-27
			TYR-23	LEU-15	ASN-31	CYS-19
			TYR-23	CYS-17		
			ASN-31	CYS-19		
			ASN-43	CYS-5		
			ASN-43	VAL-6		
			ASN-43	TRP-7		
Kalata B1			TRP-30	PRO-4	ASN-40	GLU-8
			THR-34	PRO-4	ASN-31	THR-9
			ASN-35	CYS-6	ASN-312	SER-23
			ASN-35	GLY-7		
			ASN-312	TRP-24		
			GLN-322	VAL-5		
Varv peptide E			TYR-23	THR-13	ASN-312	THR-13
			ASN-312	ASN-15	ASN-312	ASN-15
			THR-34	GLY-18	ASN-31	SER-20
			ASN-31	CYS-19	ASN-43	ASN-29
			ASN-31	SER-20		
			GLU-39	ASN-29		
			ASN-40	ASN-29		
			TYR-23	GLY-12		
Palicourein			THR-344	TYR-15	ASP-349	THR-6
			SER-262	ARG-26	ASN-272	ARG-8
			SER-262	ASP-28	ASP-349	ARG-8
			ASP-274	THR-6	ASN-136	LYS-32
			LYS-345	CYS-3	THR-276	GLU-5
					LYS-345	CYS-3
					THR-347	GLU-5
Vhl-1			TRP-30	CYS-1	ASN-40	THR-13
			TRP-30	GLY-2	GLU-311	ASN-22
			ASN-31	CYS-1	GLN-307	LYS-22
			THR-34	CYS-1	GLU-311	TYR-26
			GLN-322	LEU-27	ASN-40	THR-13

			ARG-339	GLY-2	GLU-311	ASN-22
			GLU-311	TYR-26		
			SER-313	SER-31		
Griffithsin	CYS-115	LEU-46	PRO-120	ASN-45	CYS-123	ASN-45
			GLN-121	ASN-45	SER-152	THR-49
			CYS-115	LEU-46	GLU-153	THR-49
			SER-152	THR-49	TYR-479	THR-51
			GLU-153	THR-49	ASP-453	ARG-145
Cycloviolacin VY1	ALA-28	CYS-19	ASN-312	ILE-8	ASN-43	SER-18
			ASN-35	VAL-14	ASN-31	CYS-19
			ASN-31	CYS-19	ASN-31	CYS-19
			ASN-31	CYS-19	TYR-23	LYS-22
			ASN	GLY-28	ASP-337	LYS-22

Molecular dynamics simulations

The ACE2-peptide interactions were further evaluated via molecular dynamics simulations analysis to examine the fluctuation of the respective amino acids in the individual complex and their respective conformational stability (Jamroz *et al*. 2013). Ten (10) different models were predicted by the exploratory simulation per run and the first model of each was selected based on its best structural heterogeneity, and stability. Significant changes were noticed in the structural flexibility of ACE2 investigated based on the root mean square fluctuation (RMSF). This is possibly due to the interaction of the peptides with the interface of the receptor when compared to the wild-type (Figure 3). The contact map presents the interaction interface between all the atoms in the wild-type ACE2 and the complexes. All the peptides, except Vhl-1 and varv peptide E showed residues with comparatively higher RMSF values than the wild-ACE2, thus indicating the binding of circulin A, kalata B1, palicourein, griffithsin, and cycloviolacin VY1could induce flexibility in further ACE2. The associated lower RMSF values computed from the bindings of Vhl-1 and varv peptide E with ACE2 could suggest induced limited fluctuation of complexes in the course of the simulation process. Thus, the binding of circulin A, kalata B1, palicourein, griffithsin, and cycloviolacin VY1 could influence the reliability of ACE2 secondary structure and subsequently the functional properties of the receptor and its innate capability to bind the coronavirus spike protein. This observation could substantiate use of peptides as better therapeutic agents over small molecule-based on their high target specificity and potency (Zompra *et al*., 2009; Vlieghe *et al*., 2010; Craik *et al*., 2013; Holohan *et al*., 2013).

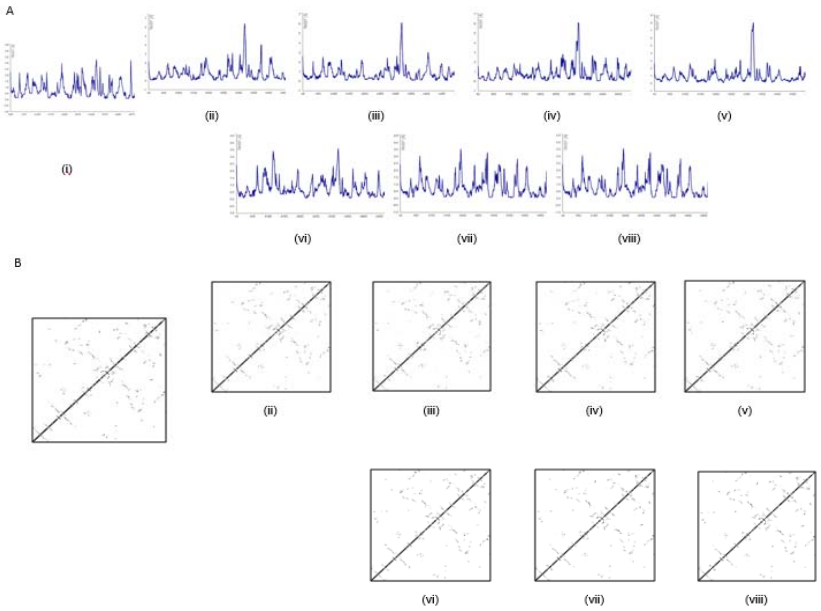

Figure 3: Molecular dynamic simulations representing (A) Fluctuation plot and (B) Contact map of (i) Wild-type ACE2 and complex of ACE2 with (ii)Circulin A, (iii) Kalata B1, (iv) Varv peptide E, (v) Palicourein, (vi) Vhl-1, (vii) Griffithsin, (viii) Cycloviolacin VY1

Conclusion

The peptides considered in this study especially Kalata B1, Vhl-1, and Cycloviolacin VY1 as well as Varv peptide E have the abilities to modulate ACE2 which might be due to their high cyclic content compared to others. These peptides could play significant roles as modulators of ACE2 and could be repositioned as anti-viral agents to prevent the entry and further replication of SARS-CoV-2. However, synthesis and standardization of the selected peptides with their corresponding experimental evaluations are recommended further studies.

Acknowledgements

The support provided by the staff of Department Biological Sciences, College of Natural and Applied Sciences, McPherson University, Seriki Sotayo, Ogun State, Nigeria is well appreciated.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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الدراسات النظرية للببتيدات ذات الأصل النباتي التي تستهدف الإنزيم المحول للأنجيوتنسين البشري المرتبط بالإنزيم carboxypeptidase

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ملخص

خلفية

إن قابلية تعرض الرئتين والأمعاء والقلب والكلى لغزو **SARS-CoV-2** تعتمد على التعبير العالي عن إنزيم **carboxypeptidase** المحول للأنجيوتنسين **(ACE2)** على السطح الخارجي للخلايا في هذه الأعضاء .يؤكد هذا الوضع الواضح للتفاعل بين بروتينات ارتفاع **SARS-CoV-2** و **ACE2** على أهمية مستقبلات **ACE2** في انتشار فيروسات كورونا .بحثت هذه الدراسة في إمكانات الارتباط لبعض الببتيدات النباتية المختارة **(Circulin A ، kalata B1 ، Varv peptide E ، Palicourein ، Vhl-1 ، griffithsin ، cycloviolacin VY1)** إلى **ACE2** كنهج تنبؤي في منع غزو **.SARS-CoV-2**

أساليب

تم استرداد الببتيدات من قاعدة بيانات الببتيد المضاد للميكروبات وتم التنبؤ بخصائصها الفيزيائية والكيميائية باستخدام أداة **.ProtParam** تم حساب وضع الربط والطاقات الحرة الملزمة من خلال خوادم **HawkDock** بينما تم تقييم المرونة الهيكلية واستقرار مجمعات **ACE2-peptide** عبر خادم**.CABS-flex 2.0**

نتائج

لوحظ أن درجات الربط للببتيدات تجاه **ACE2** أظهرت ارتباطات ارتباط جيدة مع **griffithsin** الذي حصل على أفضل درجة ربط من خلال رتبة **Hawkdock** بينما حصلت **kalata B1** على أقل درجة ربط .أظهر تحليل الميكانيكا الجزيئية / منطقة سطح الولادة المعممة أن الطاقة الحرة الملزمة تتراوح **39.99-** و **3.96-** كيلو كالوري / مول مع **Vhl-1** الذي يحتوي على أعلى طاقة حرة و **palicourein**الذي يحتوي على أقل طاقة حرة

الاستنتاجات

تشير نتائج الدراسة إلى أن الببتيدات النباتية المختارة وخاصة **kalata B1** و **vhl-1** و **cycloviolacin VY1** يمكن أن تكون مُعدِّلات واعدة لـ **ACE2** وتمنع ارتباط المجال **S1** لبروتين **SARS-CoV-2 S** وما يترتب على ذلك من خلايا خلوية .دخول**.SARS-CoV-2**

الكلمات الدالة: الببتيدات ، الإنزيم المحول للأنجيوتنسين 2 ، ألفة ملزمة ، إمكانية التعديل ، سارس-كوف.

تاريخ استلام البحث 2020/6/9 وتاريخ قبوله للنشر 2021/4/17.

Jordan Journal of Pharmaceutical Sciences, Volume 14, No. 2, 2021

Influence of Dexamethasone on Pharmacokinetic Parameters of Cyclosporine in Rabbits

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ABSTRACT

The present study was designed to investigate the presence of significant pharmacokinetic (PK) interaction between Dexamethasone (DEX) at different concentrations on Cyclosporine (CsA) PK parameters in healthy male rabbits. The rabbits were selected and divided into three groups: Control group (n=6) received orally CsA solution (7.5 mg/kg/day) prepared from soft gelatin capsule for five days and on the fifth day, serial blood samples were withdrawn from marginal ear vein of rabbits at different time intervals post-dosing. In the first and second groups, rabbits were given orally (7.5 mg/kg/day) CsA solution concomitantly with DEX at two doses (0.33 and 0.66 mg/kg/day), respectively. On the fifth day of administration, each test group's serial blood samples were collected for over 24 hours as in the control group. Different PK parameters of CsA for the three groups were determined using non-compartmental analysis. It was observed that, there were statistically insignificant differences between control and test groups when co-administered with DEX at both concentrations. The present study results demonstrated that concurrent administration of DEX at both concentrations had not influenced the PK parameters of CsA.

Keywords: cyclosporine, dexamethasone, drug-drug interaction, pharmacokinetic parameters.

INTRODUCTION

CsA is a calcineurin inhibitor and potent immunosuppressive agent, which significantly impacts organ transplantation [1]. It has considerably improved the first and second-years graft survival rates and decreased morbidity in kidney, liver, heart, lung, and pancreas transplantation. In addition to that, several studies have supported the efficacy of CsA in preventing graft-versus-host disease in bone marrow transplantation [2]. CsA is extensively metabolized in the liver by CYP3A4 system [3]. Sustained and clinically significant drug-drug interactions (DDIs) can occur during long-term therapy. Thus, the co-administration of multiple drugs with CsA could increase the risk of treatment failure, nephrotoxicity, and other adverse effects [4].

DDIs are one of the commonest causes of medication errors in developed countries, mainly in the elderly due to poly-therapy, with a prevalence of 20-40% [5]; thus, Poly-therapy increases the complexity of therapeutic management and the risk of clinically significant DDIs which can both induce the development of adverse drug

Received on 3/6/2020 and Accepted for Publication on 30/4/2021.

reactions or reduce the clinical efficacy [6]. CsA soft gelatin capsule (Neoral®) is a modified micro-emulsion formulation with uniform and slightly increased bioavailability compared to (Sandimmune®) [7, 8]. After oral administration of CsA (Neoral®), the time to peak blood concentration (t_{max}) is 1 to 2 hours (h), the elimination of CsA is primarily biliary with only 6% of the dose (parent drug and metabolites) excreted in urine, the disposition of CsA from blood is generally biphasic with a terminal half-life ($t_{½}$) approximately 8.4 h (range 5 to 18 h) [9]. Dexamethasone (DEX) is a synthetic adrenocortical steroid agent used in treating chronic inflammatory and autoimmune diseases and is also effective as an antiemetic agent in cancer chemotherapy [10]. Cytochromes P450 (CYP) is a major source of variability in drug PKs, and response [11] and CYP3A4 is the most common and versatile one [12]. Many clinically significant drug interactions result from induction or inhibition of CYP3A4 enzymes, the major drug-metabolizing enzymes mainly in the liver [13, 14].

A remarkable feature of CYP3A4 is its extreme promiscuity in substrate specificity and cooperative substrate binding, which often leads to undesirable DDIs and toxic side effects. Owing to its importance in drug development and therapy, CYP3A4 has been the most extensively studied [15]. This study aims to investigate the presence of clinically significant PK interaction between DEX at different concentrations on CsA (A narrow therapeutic index drug) PK parameters by using healthy male rabbits as an animal model.

1. MATERIALS AND METHODS

2.1 Animals

Several experimental trials were performed on 18 New Zeland strains of adult male rabbits weighted (3.1-3.4 kg) and aged 8-10 months were enrolled in this study. The Research and Ethics Committee approved animals of the Experimental Animal Care Facility, College of Pharmacy, Al-Azhar University of Gaza (AUG), Palestine. The rabbits were selected randomly and divided into three groups (six for each group). All rabbits were kept under standard laboratory conditions in a 12-hour light/dark cycle at 25°C ± 2°C provided with pellet diet with water *ad libitum* and were fasted overnight before the experiments.

2.2 Study design and blood sampling

In an in-vivo drug-drug interaction, a randomized designed study was conducted in eighteen healthy male rabbits. The rabbits were selected and divided into three groups: Control group (n=6) received orally CsA solution (7.5 mg/kg/day) prepared from soft gelatin capsule (Neoral®) for five days, and on the fifth-day blood samples (1.5-2 mL) were withdrawn from marginal ear vein of rabbits at 0.00, 0.50, 1.00, 1.50, 2.00, 3.00, 6.00, 12.00 and 24 hr post-dosing [16]. The rabbits in the first and second groups (test groups) received orally CsA solution (7.5 mg/kg/day) and DEX concomitantly at two different doses (0.33 and 0.66 mg/kg/day), respectively. On the fifth day of the administration, serial blood samples from each group were collected for over 24 hours as in the control group. Whole blood samples in EDTA tubes were kept at (2-8) ^{o} C until analyzed (Whole blood sample is stable for up to 3 days).

2.3 Analysis of blood samples

Analysis of whole blood samples to determine the concentrations of CsA was performed at the laboratory of Medical Relief Society-Gaza using Maglumi 800 System and Maglumi 800 CsA detection kit (Shenzhen New Industries Biomedical Engineering Co., Ltd.). The Kit is based on chemiluminescent immunoassay (CLIA). It is used in hospitals for rapid CsA assaying in whole blood to monitor the CsA dose.

2.4 CsA PK and statistical analysis

PK parameters for control and test groups including C_{max}, t_{max}, K_e, $t_{1/2,}$ AUC_{0-24}, $AUC_{0-\infty}$, and MRT were determined. Both parameters (C_{max}) and (t_{max}) were directly determined from the plasma concentration versus time curves. The linear trapezoidal rule calculated the AUC0-24. The $AUC_{0-\infty}$ was determined by the following equitation: $AUC_{0-\infty} = AUC_{0-24} + Ct / k_e$, where Ct is

defined as the final measured serum concentration at time t and K_e is the elimination rate constant. The K_e was determined by the least-squares regression of plasma concentration-time data points lying in the terminal region using semilogarithmic dependence that corresponds to first-order kinetics. The $t_{1/2}$ was calculated as $0.693/K_e$. PK analysis was determined using model-independent method (Non-Compartmental Approach) WinNonlin Professional Software (Version 6.3, Pharsight Corporation, Cary, NC) and (GraphPad Prism versión 4.00; San Diego, CA, USA). Statistical methods, including descriptive analysis and Mann-Whitney test, were applied to compare the PK parameters of CsA alone (control group) or co-administered with DEX in first and second groups (test groups). (SPSS) program (version 16.0) was applied to analyze data. A statistically significant difference was considered when $P \leq 0.05$.

2. RESULTS

The DEX-CsA interaction study was carried out to determine the influence of DEX at different concentrations on the PK parameters of CsA. Whole blood concentration-time profiles of CsA in the control group (Received only CsA 7.5 mg/kg/day) and test groups (First and second groups), which received CsA concomitantly with DEX at doses (0.33, and 0.66 mg/kg/day) respectively are shown in Figure 1. The PK parameters of CsA were determined for the control and test groups, including C_{max}, t_{max}, $t_{1/2}$, K_e, AUC_{0-24}, $AUC_{0-\infty}$, and MRT. The control group's obtained PK parameters were compared with those of the first test group (Table 1) and those with the second test group (Table 2).

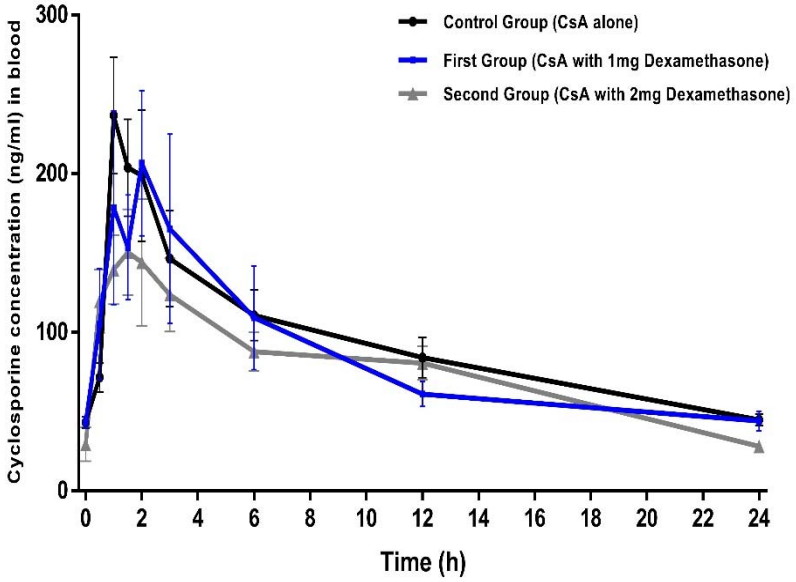

Figure 1: Plot of blood CsA concentration-time profile. Control group: Rabbits received CsA alone (7.5mg/kg/day) orally for five days, first and second groups (test groups): Rabbits received CsA (7.5mg/kg/day) concurrently with DEX (0.33 and 0.66mg/kg/day) orally for five days, respectively (n=6).

Table 1. PK parameters of CsA in control and first test group (n=6).

PK Parameters	Groups	N	Mean ± SD	P-value
C_{max} (ng/ml)	Control group	6	254.9±106.4	0.68
	First group	6	290.0±144.9	
t_{max} (h)	Control group	6	1.50±0.87	0.58
	First group	6	1.83±0.75	
Ke (h^{-1})	Control group	6	0.052±0.001	0.77
	First group	6	0.054±0.02	
$t_{1/2}$ (h)	Control group	6	13.38±1.82	0.99
	First group	6	13.29±2009	
AUC_{0-24} (ng/ml.h)	Control group	6	2221± 730	0.70
	First group	6	2009±946	
$AUC_{0-\infty}$ (ng/ml.h)	Control group	6	3069±808	0.71
	First group	6	2863±985	
MRT (h)	Control group	6	9.22±0.62	0.90
	First group	6	9.16±1.12	

Control group: Rabbits received CsA (7.5mg/kg/day) orally for five days; First group: Rabbits received CsA (7.5mg/kg/day) concurrently with DEX (0.33mg/kg/day) orally for five days; Statistical significance: $P \leq 0.05$.

Table 2. PK parameters of CsA in control and second test groups (n=6).

PK Parameters	Group	N	Mean ± SD	P-value
C_{max} (ng/ml)	Control group	6	254.9±106.4	0.51
	Second group	6	213.5±72.6	
t_{max} (h)	Control group	6	1.50±0.87	0.58
	Second group	6	1.92±0970	
Ke (h^{-1})	Control group	6	0.052±0.001	0.06
	Second group	6	0.049±0.012	
$t_{1/2}$ (h)	Control group	6	13.38±1.82	0.06
	Second group	6	18.97±5.61	
AUC_{0-24} (ng/ml.h)	Control group	6	2221±730	0.59
	Second group	6	1975±333	
$AUC_{0-\infty}$ (ng/ml.h)	Control group	6	3069±808	0.55
	Second group	6	2747±497	
MRT (h)	Control group	6	9.22±0.62	0.55
	Second group	6	8.79±0.83	

Control group: Rabbits received CsA (7.5mg/kg/day) orally for five days; Second group: Rabbits received CsA (7.5mg/kg/day) concurrently with DEX (0.66mg/kg/day) orally for five days; Statistical significance: $P \leq 0.05$.

In this study, C_{max} of CsA (control group) was 254.9±106.4 ng/mL, and t_{max} was 1.50± 0.87 h. The rabbits treated with DEX 0.33 mg/kg/day (first group) produced C_{max} and t_{max} of 290.0±144.9 ng/mL and 1.83±0.75 h, respectively. A slight increase of C_{max} and t_{max} of CsA were found when DEX (first group) was co-administered with CsA compared to the control group. The differences were statistically insignificant. A slight decrease in AUC_{0-24} and $AUC_{0-\infty}$ was manifested in the first test group in comparison to control, but it was statistically insignificant ($P \geq 0.05$). Other PK parameters, including $t_{1/2}$ and K_e were also insignificant (Table 1). Blood concentration-time profiles of CsA were comparable for control, and second test groups (Figure 1).

PK parameters of CsA (C_{max}, t_{max}, ke, $t_{1/2}$, AUC_{0-24}, $AUC_{0-\infty}$ and MRT) were unaffected ($P \geq 0.05$) by co-administration of DEX at a concentration of 0.66 mg/kg/day (second group) as shown in (Table 2).

3. DISCUSSION

Most immunosuppressive agents have narrow therapeutic indexes, so dosing most of the immunosuppressive agents is applied under careful monitoring of their blood concentrations. Knowing the potential factors that can modify immunosuppressive therapy and pharmacokinetics and metabolic drug interactions can decrease the fluctuation of immunosuppressant blood concentrations [17]. CsA is extensively metabolized in the liver by the CYP3A4 system, a member of the CYP450 family of oxidizing enzymes [3,12]. Sustained and clinically significant DDIs can occur during long-term therapy with CsA. The co-administration of multiple drugs with CsA could result in graft rejection, renal dysfunction, or other undesirable effects. Potential CsA drug interaction is of great clinical importance [4]. Drugs affecting CYP3A4 metabolic activity are a possible candidate for such interactions [17]. Synthetic glucocorticoids as dexamethasone or prednisolone are known substrates and inducers of CYP3A enzymes [18]. The regulation of these enzymes, particularly CYP3A4, has been extensively studied [19]. In vitro studies of DEX in cultures of human hepatocytes showed potent CYP3A4 inducing effects. In vivo studies had shown that co-administration of DEX with some drugs as phenytoin resulted in reduced plasma level of the drug. When DEX was discontinued, plasma concentration increased by 300% and increased CYP3A4 activity in healthy volunteers and human hepatocyte cultures [20-25]. The conducted study of DEX-CsA interaction showed statistically insignificant differences in PK parameters when CsA was administered alone or in combination with DEX given at two different doses. Similar results were obtained in another research work realized by Villikka and collaborators who studied the PK of triazolam (CYP3A4 substrate) when co-administered with DEX (4-day course of 1.5 mg dexamethasone daily) [26]. The impact of an enzyme inducer like DEX increases gradually over time. The effect of DEX doses on PK of tacrolimus was manifested after three months of treatment after one month [27]. This could explain the obtained results since DEX was co-administered with CsA for five days. The possibility that higher doses of dexamethasone could induce CYP3A4 and thereby cause clinically significant drug interactions cannot be excluded. This is particularly relevant in cancer chemotherapy as many anticancer agents are CYP3A4 substrates, and DEX as an antiemetic agent is used at high doses [28].

In conclusión, the present study results demonstrated that concurrent use of DEX at the examined regimen with CsA had not influenced PK parameters of CsA. Furthermore, PK studies of CsA using DEX at higher doses and for a longer duration and other clinically relevant CYP inducers or inhibitors are advised.

Conflict of interest

The authors proclaim no conflict of interest.

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تأثير دواء الديكساميثازون بجرعات مختلفة على حركية دواء السيكلوسبورين

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ملخص

تم تصميم هذه الدراسة للتحقيق في وجود تفاعل حركي دوائي بين ديكساميثازون **(DEX)** بتركيزات مختلفة على حركية دواء السيكلوسبورين **(CsA)** في الأرانب الذكور السليمة صحيا. تم اختيار الأرانب وتقسيمها إلى ثلاث مجموعات: المجموعة الضابطة (ع = **6**) تلقت محلول السيكلوسبورين عن طريق الفم (**7.5** ملغم / كغم / يوم) بعد تحضيره من كبسولة الجيلاتين الطرية لمدة خمسة أيام وفي اليوم الخامس تم سحب عينات الدم التسلسلية من الوريد الطرفى لاذن الارانب في فترات زمنية مختلفة بعد الجرعات. أعطيت الأرانب في المجموعتين الأولى والثانية عن طريق الفم محلول السيكلوسبورين **7.5**) مجم / كجم / يوم) و ديكساميثازون بالتزامن مع جرعتين (**0.33** و **0.66** مجم / كجم / يوم) على التوالي. في اليوم الخامس من اعطاء الدوائين ، تم جمع عينات دم متسلسلة لكل مجموعة اختبار على مدى **24** ساعة كما في المجموعة الضابطة ومن ثم تم تحديد الحركيات الدوائية للسيكلوسبورين في مجموعات الضبط والاختبار. لوحظ أنه كانت هناك اختلافات غير مهمة احصائيا بين مجموعات الضبط والاختبار عند اعطاء محلول السيكلوسبورين مع الديكساميثازون بالتركيزين المذكورين اعلاه مما يشير إلى أن الديكساميثازون لا يؤثر على حركية دواء السيكلوسبورين.

الكلمات الدالة: السيكلوسبورين. الديكساميثازون. التفاعلات الدوائية . المعلمات الحركية الدوائية.

تاريخ استلام البحث 2020/6/3 وتاريخ قبوله للنشر 2021/4/30.

Jordan Journal of Pharmaceutical Sciences, Volume 14, No. 2, 2021

Pancreatic Lipase Inhibition by Edible Plants Used in Three Middle East Countries: A Mini-Review

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ABSTRACT

Obesity is considered a serious prevalent disease that is accelerating at an alarming rate. It has drawn worldwide attention and many approaches have been suggested to reduce dietary lipid hydrolysis and absorption. One of the substantial approaches is to inhibit pancreatic lipase (PL) that is the major responsible enzyme for fat digestion in the gastrointestinal lumen. Unfortunately, till now, the only pharmaceutical drug approved for obesity management is "Orlistat", which has many unpleasant side effects. During the last decade, a massive effort has been dedicated to discovering safe lipase inhibitors from natural sources to avoid undesired drawbacks. The Middle East region is well known for its highly diverse flora with vast potential medicinal value. In this review, we try to provide an overview of the edible plants with potent anti-lipase activities and comprise a number of common ingredients in the Middle Eastern cuisine.

Keywords: Pancreatic lipase; edible plants; obesity; Jordan, Palestine, Tunisia, Middle East.

INTRODUCTION

According to WHO definition, obesity is defined as an abnormal or excessive accumulation of fat that forms a risk to health. It has become a worldwide serious challenge since the overweight rate has been increasing dreadfully. A recent fact sheet from WHO reported that in 2016, out of the 7.5 billion world population then, more than 1.9 billion adults, were overweight and more than 650 million were obese.[1] In addition, the prevalence of obesity has been increasing dramatically in children and adolescents in both developed and developing countries.[2] Indeed, over 340 million children and teenagers aged 5-19 were overweight or obese in 2016. Moreover, 41 million children under the age of 5 were overweight or obese.[3]

The pathogenesis of obesity is multifactorial. It comprises both modifiable and non-modifiable risk factors. One of the major risk factors is a chronic surplus in the daily intake of calories to the maximum fat burning capacity of the body. Such negative balance is nourished by a triad of unhealthy lifestyle habits, high-fat diet and inadequate physical activity. The clinical outcomes of

Received on 12/7/2020 and Accepted for Publication on 30/4/2021.

obesity are diverse ranging from no symptoms to complications with tremendous risks for morbidity and mortality such as type 2 diabetes, hypertension, coronary heart diseases, dyslipidemia, atherosclerosis, gall bladder and liver diseases and a number of cancers.[4] Obesity has also been linked to infertility and menstrual dysfunction and anovulation.[5]

Obesity represents a heavy burden on the healthcare systems and national expenditures worldwide. In USA for example, obesity and its associated chronic diseases were estimated to have direct healthcare costs of more than $480 billion and more than $1.24 trillion in indirect cost of work loss in 2016.[6] Despite of expansions in public awareness campaigns against obesity and regulatory changes, the prevalence rates globally are still rising. Hence, the need for new treatment modalities and novel pharmacologic options has never been greater.

A plausible pharmacologic target to manage obesity is reducing the digestion and absorption of dietary fat in the digestive tract. Lipases are enzymes that catalyze the breakdown of ester bond in triglycerides to form monoglycerides and free fatty acids, the more absorbable forms, which will then be stored in adipose tissues, leading to obesity.[7] Pancreatic lipase (PL) is the key enzyme of fat digestion accounting for the hydrolysis of 50-70% of dietary fat.[8] Not surprisingly, it drew the attention of researchers to develop natural or synthetic drugs to selectively inhibit the enzyme and halt its actions. This latter is an intriguing approach to help conquer obesity ameliorate many of its related public health concerns.

Nonetheless, pharmaceutical drugs combating obesity are still limited on the market due to non-negligible adverse effects of drugs in development. Therefore, the need for therapeutic remedies with safe, non-toxic ingredients is highly sought after. Such remedies have been explored in complementary and alternative medicine (CAM) products worldwide. According to World Health Organization (WHO), around 21,000 plant species can be used as medicinal plants and around 7000 compounds used in modern medicine come from medicinal plants.[9] Moreover, around 80% of the population of developing countries use folk medicine to meet their primary health care needs and around 25% of prescriptions in USA contain one or more plant products.[9]

The current article, best to our knowledge, is the first review of the pancreatic lipase (PL) inhibition activity of edible plants from the Middle East region aiming to provide promising strategies of reducing obesity.

HUMAN PANCREATIC LIPASE STRUCTURE

Different types of lipases (triacylglycerol hydrolases) are members of human lipase superfamily such as endothelial, lingual, hepatic, lipoprotein, lungs, kidney, skeletal muscles, adipose tissue, placenta, gastric and pancreatic lipases.[10]

The three-dimensional structure of human pancreatic lipase was first determined and published in Nature journal.[11] X-ray crystallography was used to determine its structure and the results showed that it consisted of a single chain glycoprotein, with 449 amino acids. The primary structure was deduced using sequencing complementary DNA clones. The structural results have proved that Ser152 was the nucleophilic residue essential in the enzyme activity as the catalytic function was lost after a chemical alteration of this residue. Ser152 is a part of an Asp-His-Ser triad. The previous finding was emphasized by another study which had used X-ray technique to support the identification of the Ser 152, His 263, Asp 176 catalytic triad.[12] Bioinformatics studies revealed the tertiary structure of human pancreatic lipase with α-helices, β sheets and random coils.[13] A surface lid or flab covers the presumptive active site which makes it unreachable by solvents. This flap is re-oriented upon activation.

LIPASE MECHANISM OF ACTION

Lipid metabolism starts with partial hydrolysis of ingested dietary fat by lingual lipase and stomach gastric

lipase to convert triacylglycerol into diacylglycerol and free fatty acids. The partially digested fat will be emulsified by the bile salts, secreted from the duodenum, into small fat micelles to increase the surface area and enzymatic hydrolysis efficiency. Triglycerides occupy the center of the droplet while the surface is covered with polar lipids, fatty acids, phospholipids, cholesterol and bile salts.

Hydrolytic action of gastric lipase continues in the duodenum in synergy with pancreatic lipase to convert lipids into monoglycerols and free fatty acids. Eventually, the fat micelles will be absorbed by intestinal enterocytes. Pancreatic protein co-lipase is a cofactor that facilitates hydrolytic activity of the pancreatic lipase and stabilizes its conformation.[10]

PL MECHANISM OF INHIBITION

The mechanism of PL inhibition is based on impairing the catalytic role of PL in triglyceride hydrolysis and inhibition of fat breakdown into monoglyceride and fatty acids.[14]

NATURAL AND SYNTHETIC LIPASE INHIBITORS

Extensive research in anti-obesity effects of natural and synthetic products has been profoundly progressing. A meta-analysis review of data spanning the years 2000-2018 was published in 2019, revealing that most of the plants exhibit their anti-obesity mode of action through regulating lipid profile and inhibiting pancreatic lipase.[15] There is a large pool of plant phytochemicals that have potent PL inhibitory actions, such as saponins, terpenes, phenolics, glycosides, alkaloids, carotenoids and polysaccharides.[16,10,17,18] On the other hand, robust studies on synthetic lipase inhibitors such as phosphonates, boronic acids and fats analogues have been carried out.[19] The most potent synthetic lipase inhibitor reported was 2-[(2-oxohexadecanoyl) amino]-1-[[(2-oxohexadecanoyl)-amino] methyl] ethyl decanoate that decreased the activity of human pancreatic and gastric activities to the half at 0.076 and 0.020 surface molar fractions, respectively.[10] Other studies have documented the promising roles of pharmacological agents like novel fluoroquinolones and triazolofluoroquinolones in lipase inhibition and obesity management.[19-22] In addition, a recent study has shown, for the first time, the fundamental potential of the agrochemical quinclorac as a potent synthetic and safe lipase inhibitor via *in vivo* bioactivity experiments.[23] So far, Orlistat is the first and only drug that was approved by US FDA in 2000 and introduced to the market under the name Xenical and later approved as an over-the–counter drug.[24] It is considered a semi-synthetic PL inhibitory drug from microbial origin, as it is a saturated derivative of lipstatin, the natural, irreversible lipase inhibitor from *Streptomyces toxytricini*.[25] It inhibits gastric and pancreatic lipases in the gastrointestinal tract, by competing with dietary fat molecules for the active sites of lipases, blocking fat hydrolysis pathway, thus eliminating fat absorption and storage inside adipocytes. So far, orlistat is the only anti-obesity drug on the market. Nevertheless, it still has many undesirable side effects, such as oily stools, diarrhea, abdominal pain and fecal spotting.[26]

PL INHIBITORS IN EDIBLE PLANTS

Many of the culinary herbs and plants used in the Middle East have been widely studied and claimed to have medicinal benefits. Indeed, in the last decade,[27] numerous screening studies of edible plants from Middle East region have been conducted to investigate their potential anti-lipase activity.

Jordan, Palestine and Tunisia are characterized by their high diversity and the abundance of edible flora that have substantial potential therapeutic roles as complementary and alternative medicines.

Jordan

Bustanji and colleagues (2010) studied the lipase inhibition action of *Rosmarinus officinalis* L. (Rosemary), which is a native perennial plant to Jordan that is used extensively in the Mediterranean cuisine as a cooking seasoning. It is used also in folkloric medicine, as a

treatment for dysmenorrhea,[28] respiratory disorders and hair growth stimulant.[29-30] Its anti-inflammatory and anti-oxidant effects have been studied thoroughly and it is known to have a hypo-glycemic and hypo-lipidemic dual effect. The latter effects were attributed to the presence of active phytochemicals like monoterpenes, diterpenes and the phenolics, mainly caffeic acid derivative, acids like rosmarinic acid (RA). The results of Bustanji et al revealed a significant PL inhibitory effect of rosemary extract with a low IC_{50} dose (13.8 µg/mL). The active constituents of rosemary are rosmarinic acid (RA), chlorogenic acid (CA), caffeic acid (CaA) and gallic acid (GA). They all showed inhibitory actions but the most potent one was GA with the lowest IC_{50}.[31]

Another experimental study was carried out by Issa and collaborators (2011) to investigate the effect of methanolic extract of *Lavandula angustifolia* (lavender) on diabetic dyslipidemia. This plant is native to northern regions of Jordan and is well known in this area as a folkloric medicine to diabetes. Their results showed a concentration-dependent manner inhibitory activity of lavender on PL with an IC_{50} 56.5 µg/mL. The authors inferred that this inhibitory effect could be associated with the presence of RA (IC_{50} 125.2 µg/mL) and GA ($IC_{50}$of 10.1 µg/mL).[32]

Bustanji and co-workers (2011b) have also investigated the anti-lipase activity of 23 medicinal plants, belongs to 15 plant families from Jordan, and compared their potential anti-lipase effects. Thirteen species demonstrated anti-lipase activity with IC_{50} less than 1.0 mg/mL. These were *Anthemis palaestina* Boiss. (107.7 µg/mL), *Salvia spinosa* L. (156.2 µg/mL), *Ononis natrix* L (167 µg/mL), *Fagonia arabica* L. (204.1 µg/mL), *Origanum syriaca* L. (234µg/mL), *Hypericum triquetrifolium* Turra (236.2 µg/mL), *Malva nicaeensis* All. (260.7 µg/mL), *Chrysanthemum coronarium* L. (286.1 µg/mL), *Paronychia argentea* Lam. (342.7 µg/mL), *Convolvulus althaoieds* L. (664.5µg/mL), *Reseda alba* L. (738 µg/mL), and *Adonis palaestina* Boiss (937.5 µg/mL). Some of these medicinal plants are edible.

The two most potent PL inhibitors among the aforementioned plants were *A. palaestina* and *S. spinosa*. With IC_{50}107.7 µg/mL and 156.2 µg/ml for *A. palaestina and S. spinosa*, respectively. The results were deemed pharmacologically significant in comparison with the reference drug orlistat IC50 (0.65 µg/mL) by using spectrophotometric assay to measure the PL inhibition activity.[33]

Another study from Jordan investigated the lipase inhibition activities of methanolic extracts of *Ginkgo biloba* L. (Ginkgoaceae) leaves.[34] The authors documented by virtual studies that the active phytochemical terpenetrilactones, including ginkgolides and bilobalide, fit into the active site of PL. Moreover, the experimental studies reported the PL inhibition activity of the active constituents with IC_{50} = 22.9, 90.0 and 60.1 µg/mL, respectively.[34]

In vitro and *in vivo* investigations of anti-obesity activities of another edible plant called *Crataegus aronia* L. (Rosaceae) from Jordan were conducted.[35] Crude aqueous extract of *C. aronia* was tested for its anti-lipase profiles. A significant concentration-dependent anti-lipase activity was demonstrated compared to orlistat. An *In vivo* experiment was conducted to examine the effect of the diet-supplemented with *C. aronia* crude extract on the body weight, compared with the regular diet of rats. They reported a significant decrease in the body weight of rats having the crude extract of *C. aronia,* in comparison with the control group. Their findings proposed that *C. aronia* has a substantial effect on PL in inhibition of fat digestion.

In 2015, Mohammad and co-workers virtually and experimentally examined the PL inhibitory efficacy of turmeric (*Curcuma longa* Linn.) from the family Zingiberaceae, a well- known natural antioxidant that is widely used in Mediterranean cuisines as a food additive. They concluded via the docking studies that curcumin, the active phytochemical, fits the binding site of the PL enzyme, along with anti-pancreatic lipase activity (IC_{50}

value of 7.3 µg/mL). These results support the robust use of curcumin as a promising PL inhibitor.[36]

Dietary *Salvia triloba* (Lamiaceae), known in Jordan as sage, was also studied for its lipid-lowering effects in experimental rats. Methanolic extract of the leaves exhibited a PL inhibitory potency in a dose-dependent manner and an IC_{50} of (100.80 ± 9.07 µg/mL), compared to orlistat IC_{50} (0.114 ± 0.004 µg/ml). These inhibitory effects were largely attributed to the phytochemical constituents of *S. triloba*, as flavonoids, phenolic acids and triterpenes (oleanolic acid, carnosic acid, and ursolic acid). The effect of methanolic extract of *S. triloba* on plasma triacylglyceride levels was measured in rats received high fat diet and the results revealed a marked reduction in serum triacylglyceride levels, compared to the orlistat treated group. *In vitro* results were supported by the *in vivo* findings and the authors suggested dual hypotriglyceridemic and antilipolytic properties of the sage leaves with a significant potential as an anti-obesity treatment.[37]

Palestine

A screening study from Palestine was published by Jaradat *et al*. in 2017 for PL inhibtion effects of some native medicinal and edible plants. Their results showed that *Urtica urens*, *Brassica napus*, *Portulaca oleracea* have inhibitory activity against PL. The organic and aqueous extracts of *U. urens* showed the highest anti-lipase activity with IC_{50}157 µg/mL and 157.1 µg/mL, respectively. The organic extracts of *P. oleracea* came next with IC_{50}262.03 µg/mL and the least active were the aqueous extracts of *B. napus* and *P. oleracea* that had IC_{50} 296.87 µg/mL and 417.62 µg/mL, respectively. They recommended that these three edible plants can be used as food additives in dietary industry to regulate body weight by reducing the hydrolysis and absorption of fatty food.[38]

Jaradat and co-workers conducted another study in 2017, to screen the anti-lipase activity of ten medicinal and edible plants collected from West Bank area of Palestine. The tested plants are: *Arum palaestinum* Boiss, *Crataegus azarolus* L., *Malva parviflora* L., *Taraxacum syriacum* Boiss, *Rhus coriaria* L., *Rosmarinus officinalis* L., *Psidium guajava* L., *Origanum dayi* Post, *Brassica nigra* (L.) K. Koch, *and Vitis vinifera* L. The inhibition activity was assessed by using UV-visible spectrophotometer, and it was compared to the reference drug orlistat, which had an IC_{50} value 12.38 µg/mL. Among the ten screened native plants, they found that aqueous extracts of *V. vinifera* and *R. coriaria* were the most potent inhibitors with IC_{50} values of 14.13 and 19.95 µg/mL, respectively. The organic extract of *O. dayi* was also considered a potent PL inhibitor with an IC_{50} value of 18.62 µg/mL. This study was the first in screening the lipase inhibition activity of these three potent natural inhibitors which may play a significant role in obesity prevention.[39]

A recent article was published in 2018 about the anti-obesity potential of selected species from Palestine. The authors examined 90 plant species and found that the top active species in PL inhibtion in terms of minimum IC_{50} value are *Camellia sinensis*, *Ceratonia siliqua, Curcuma longa, Sarcopoterium spinosum, Mentha spicata*. These findings promote the use of the natural resources in overweight regulation.[40]

Tunisia

An in vitro study to assess the lipase inhibitory ability of *Juniperus phoenicea* L. was conducted in 2014. Its berries are used in cooking seasoning as well as in traditional medicine in the Middle East area. The results of the study revealed a powerful inhibitory effect of *J. phoenicea* extracts against PL, compared to the positive control, orlistat. These findings are consistent with the presence of phenolic phytochemicals in *J. phoenicea* leaves.[41]

Sellami and colleagues screened different extracts from various spices used in Tunisian cuisine, besides some aromatic beverages as coffee, green and black tea that are used commonly in Tunisia. Seventy-two plant extracts

were examined *in vitro* for their anti-lipase inhibitory effects, out of which, cinnamon *(Cinnamomum verum)* and mint (*Mentha aquatic*) showed the most potent and powerful PL inhibitory activities with the IC_{50} of 45 and 62 µg/ml, respectively. The strong PL inhibitory potency of both cinnamon and mint was suggested to be due to the presence of active constituents such as polyphenols, saponins, tannins, terpenes, flavonoids and alkaloids. Moreover, the inhibition effect was irreversible in presence of bile salts and colipase. The authors suggested that these two culinary plants could be a good source for new progenitors of anti-obesity drugs.[42]

CONCLUSION

A tremendous work is carried out to find an efficient and safe pancreatic lipase inhibitor from natural origin. This research mania is driven by the ascending worldwide awareness of the pathological consequences of obesity as a life-threatening disease. Currently, orlistat (Xenical) is the only drug available on the market for obesity. It should be emphasized that obesity is not synonymous with hyperlipidemia, even though it's considered a risk factor for hyperlipidemia. The possible explanation for the scarcity of anti-obesity drugs compared to lipid lowering drugs is the fact that obesity is multifactorial. Hence, the success rate of any pharmacologic intervention is highly variable among individuals. This has discouraged major pharmaceutical companies from investing in the development of new drugs for that indication. Another limiting factor is the inability of pharmaceutical manufacturers to patent drugs extracted from natural plants since these are already found naturally without any credit to the manufacturers. A third reason is the variabilities in the constituents of natural plants from one geographic area to another which further complicates the manufacturing process and quality validation. Nonetheless, the need for an alternative treatment strategy for obesity has driven a wealth of investigations of potential natural progenitors that can be synthetically modified to create potential drug candidates. An overview of the Middle Eastern food reveals the high correlation of the consumable plants with the inhibition of fat digestion. Undoubtedly, many edible plants from the Middle East region are good candidates for the discovery of new influential and harmless lipase inhibitor drugs.

ACKNOWLEDGMENTS

The authors are grateful to the Deanship of Scientific Research / The University of Jordan, Amman, Jordan for their financial support.

Conflicts of interest

The authors declare no conflicts of interest.

Authorship

All authors contributed equally to the literature search, participated in writing the review and approved the submission.

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تثبيط انزيم الليباز البنكرياسي بواسطة النباتات الصالحة للأكل المستخدمة في ثلاث دول في الشرق الأوسط: مراجعة مصغرة

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ملخص

تعتبر السمنة من الأمراض الخطيرة المنتشرة والتي تتسارع عالميا بوتيرة تنذر بالخطر. و هذا الانتشار السريع لفرط السمنة نبه المجتمعات في جميع أنحاء العالم لايجاد حلول جذرية للسمنة لما لها من مخاطر كبيرة على صحة الافراد و المجتمعات. و تم اقتراح العديد من من الأساليب لتقليل التحلل المائي للدهون الغذائية للحد من امتصاصها. و من اهم هذه المقترحات عمل تثبيط للأنيم الليباز البنكرياسي **(PL)**. وهو الإنزيم الرئيسي المسؤول عن هضم الدهون في تجويف الجهاز الهضمي. و لكن للاسف الدواء الصيدلاني الوحيد المسجل لهذه الغاية هو "أورليستات" ، و الذي له العديد من الآثار الجانبية. خلال العقد الماضي ، تم تكريس جهد كبير لاكتشاف مثبطات جديدة لأنزيم الليباز البنكرياسي الآمنة من المصادر الطبيعية لتجنب الآثار الجانبية غير المرغوب فيها. تشتهر منطقة الشرق الأوسط بنباتاتها المتنوعة ذات القيمة الطبية الكبيرة المحتملة لعلاج العديد من الامراض المزمنة. في هذه المراجعة ، نحاول تقديم لمحة عامة عن النباتات الصالحة للأكل ذات الأنشطة القوية المضادة لليباز البنكرياسي وتشتمل على عدد من المكونات الشائعة في مطبخ الشرق الأوسط.

الكلمات الدالة: الليباز البنكرياسي، النباتات التي تؤكل، السمنة، الاردن، فلسطين، تونس، الشرق الاوسط.

تاريخ استلام البحث 2020/7/12 وتاريخ قبوله للنشر 2021/4/30.

Phytochemical profiling and antibacterial activities of extracts from five species of Sumatran lichen genus *Stereocaulon*

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ABSTRACT

In continuing the inventory and investigation of Sumatra lichen, a collection of five species from the genus *Stereocaulon* has been carried out. In metabolite profiling analysis, the four main compounds, i.e. atranorin, MOC, lobaric acid, and stictic acid, were rapidly isolated as marker compounds by chromatography methods from *S. halei* and *S. montagneanum*. Then, the extracts from each species were analyzed using HPTLC densitometry measured at five wavelengths of 220, 254, 280 320, and 365 nm. Atranorin and MOC are two compounds found in all five *Stereocaulon* species tested. Subsequently, atranorin quantification was done by densitometric scanning at 254 nm from the ethyl acetate extract of each *Stereocaulon* species. The highest atranorin concentration was detected from *S. graminosum* (325,498 mg/g extract) and equivalent to 0.065 mg/g dried whole thallus, while the lowest was from *S. verruculigerum* (23,356 mg/g extract) and 0.023 mg/g dried whole thallus. Furthermore, all extracts and main compounds resulting from the isolation were evaluated for their antibacterial activity by the microdilution method. Ethyl acetate and acetone extracts from *S. massartianum* (1) showed the highest antibacterial activity against *E. faecalis* (MIC = 1.25 mg/mL).

Keywords: Atranorin , HPTLC-densitometry , Lichen , Metabolite profiling , *Stereocaulon*.

1 INTRODUCTION

The genus *Stereocaulon* Hoffm. (Stereocaulaceae, Lecanorales, Ascomycota) is an interesting genus from lichen which is found throughout the world. The morphology of the *Stereocaulon* genus consists of the crustose type primary thallus and fruticose type secondary thallus. The primary thallus in most species of *Stereocaulon* is disappeared at a very early stage of development. In the secondary thallus, there are several important parts such as pseudopodetia which show persistent phyllocladia (or phyllocladioid branchlets), apothecia as a sexual organ that contains spores, and in most species cephalodia which contain cyanobacteria (Nostoc, Rhizonema or Stigonema)[1].

Although the *Stereocaulon* genus is grow scattered throughout the world, its scientific study is still limited, thus, it needs to be deeply researched to uncover its potential. Some famous lichenologists have described this genus, such as Nylander in his Synopsis Lichenum[2], Riddle[3], Magnusson[4], Dodge[5], Johnson[6], Duvigneaud[7], Lamb[8-10], Boehkout[11], and McCarthy[12]. Also, secondary metabolite profiles of the genus *Stereocaulon* play an essential role in the identification of lichen as Duvigneaud[7] and Lamb[9,10] have done using microchemistry. Until now, several thorough phytochemical studies of Stereocaulon species have been carried out[13,14], and among the 40 species studied, the depsides, depsidone, dibenzofurans, diphenyl ethers groups were isolated for the most

Received on 2/4/2020 and Accepted for Publication on 20/5/2020.

representative secondary metabolites for lichens[1].

In Sumatran lichen exploration, we have carried out several phytochemical studies on some genera such as Cetrelia and Usnea[15,16] and focused more on the genus *Stereocaulon*, such as *S. halei, S. montagneanum,* and *S. graminosum*[17-19]. In the surveys conducted in the period 2015-2016 in the mountains and highlands of West Sumatra and Jambi province, Indonesia, we collected five species of the genus *Stereocaulon*. This paper described a phytochemical approach with rapid isolation of the main depside compound (atranorin, **1**), monoaromatic compound (Methyl-β-orcinol carboxylate, MOC, **2**) and depsidones (stictic acid (**3**) and lobaric acid, **4**) from *S. halei* and *S. montagneanum* (Figure 1). These compounds were used as marker compounds in the metabolite profiling analysis with the HPTLC-densitometry. We also conducted the determination of atranorin concentration with the densitometry approach at 254 nm of the extracts, and this was done for the first time. Then, an antibacterial evaluation was carried out for each extract and isolated compound against four pathogenic bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 12228, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853) with the microdilution method.

2 *MATERIALS AND METHODS*

2.1 Lichen material

Five lichen species were collected in the highlands and mountains in the regions of West Sumatra and Jambi province, Indonesia. *S. halei* and *S. massartianum* were collected in different locations and altitudes, so the number of the sample analyzed was seven samples. Some ecological parameters are recorded in Table 1. We confirmed our identification by sending all samples to Harrie Sipman (Berlin Museum), and specimen vouchers were deposited at the Biota Sumatran Laboratory, Andalas University, West Sumatra (Indonesia) with the reference numbers cited in Table 1.

2.2 Instrumentations

Some equipment was used for the characterization of compounds. The melting point was measured on a Fisher Melting Point apparatus. UV spectra were performed on a Shimadzu 1700 spectrophotometer. FTIR spectra were run on a Perkin Elmer FTIR spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded at 500 and 125 MHz, respectively, on a Jeol 500 MHz NMR spectrometer using $CDCl_3$ and DMSO-*d6* as solvents. The HPTLC plate was densitometrically scanned with CAMAG TLC scanner 4. Data acquisition and processing were recorded using the software winCATS version 1.4.7.

2.3 Reagents and materials

All the chemicals and reagents used in this study were analytical grade and were purchased from Merck. Chromatographic separation was performed using vacuum liquid chromatography on silica gel (Merck 0.063-0.200 mm). HPTLC aluminium sheets 20x20 cm, Merck KGaA, Darmstadt Germany, Cat.no.1.05548, were eluted using two standard solvent systems[20] that is toluene/acetic acid (85:15) (C); toluene/EtOAc/formic acid (139:83:8) (G). Visualization of plates was carried out under UV light (254 and 365 nm) and then sprayed with anisaldehyde $H2SO_4$ (v/v) reagent followed by heating at 110ºC.

2.4 Isolation marker compounds

The Compound **1**, **2**, **3** and **4** as marker compounds were isolated from *S. halei* (collected from Mount Singgalang) and *S. montagneanum* (collected from Sirukam, Solok) based on previous work. These two species were chosen based on their sufficient biomass. Air-dried whole thallus powder (500 g) from *S. halei* were macerated successively with ethyl acetate, acetone, and methanol (2x1 L). The obtained ethyl acetate extract was filtered and evaporated to half, and afterward, it was left for 24 hours at room temperature to precipitate crude crystalline. The crude crystal was recrystallized with ethyl acetate repeatedly and 6.3 g compound **1** (1.26% w / w) was obtained. The ethyl acetate filtrate (5 g) was chromatographed on a vacuum liquid chromatography

silica gel column (100 g) eluted with a step gradient polarity solvents consisted of n-hexane–EtOAc (100:0→0:100) as the mobile phase, and lastly washed with methanol. The eight subfractions acquired (E1-E8) were monitored by TLC and subfraction E4 and E6 indicate the spot estimated as compound **2** and **4**, respectively. Both subfractions were chromatographed over Sephadex LH 20 column chromatography to yield compound **2** (150 mg) from subfraction E4 and Compound **4** (90 mg) from subfraction E6.

Meanwhile, 500 g air-dried whole thallus powder from *S. montagneanum* were extracted by step gradient polarity starting from ethyl acetate, acetone, and methanol (2x500 ml). The acetone liquors are concentrated to half and allowed to stand for 24 hours at room temperature. The resulting precipitation was then filtered and washed with acetone:methanol (1:1) and obtained 300 mg compound **3**. The physical data, IR, melting point, ^{1}H and ^{13}C NMR data of all isolated compounds were analyzed.

2.5 Preparation of lichen extracts and standard solution for metabolite profiling

Approximately 50 g of seven air-dried whole thallus of *Stereocaulon* species were macerated successively with ethyl acetate, acetone, and methanol (2 times × 250 ml), and each extract was concentrated *in vacuo*. Preparation of each extract solution (EtOAc, Acetone, and methanol extracts) was carried out by weighing each extract accurately and dissolved with an appropriate solvent to obtain 10 mg/ml concentration of each sample.

The marker compounds (**1, 2, 3** and **4**) that were isolated before were weighed precisely as much as 10 mg each and dissolved in ethyl acetate and acetone with the volume was made up to 10 ml in a volumetric flask.

A volume of 2 µl of marker compounds and each extract solution were applied to the chromatographic plate (20 x 10 cm) using CAMAG Nanomat 4 and was developed at room temperature in flat bottom chamber (20 x 20 cm) with solvent system toluene:EtOAc:formic acid (139:83:8). After development, HPTLC plates were heated at 105ºC and scanned using the scanner at 5 wavelengths 220, 254, 280, 310, and 365 nm. Spot identification was based on its Rf value, the maximal wavelength, and visual evaluation after spraying anisaldehyde sulfuric reagent.

2.6 Preparation of lichen extracts and standard solution of atranorin (1) for quantitative analysis

As well as the sample preparation for the metabolite profiling, the quantitative analysis sample preparation was done by weighing 50 g of each air-dried whole thallus of **Stereocaulon** species and then was extracted by 250 ml of ethyl acetate solvent for two days, filtered and evaporated in vacuo. Afterward, each dried extract was weighed 10 mg and dissolved with ethyl acetate-acetone (ratio 3: 2) to the limit marking on a 10 ml volumetric flask.

Atranorin (**1**) standard solution is made by weighing 10 mg atranorin dissolved in 10 ml acetone which will then be diluted to make a calibration curve.

Preparation of calibration solutions was done by weighing 10 mg of atranorin (**1**) dissolved in 10 mL of acetone; solutions with concentrations of 250, 200, 150, 100, 50 and 25 µg/mL were prepared by dilutions of the stock solution. Atranorin (**1**) calibration curves are calculated by plotting the area of the concentration from the standard solution.

Atranorin (**1**) as standard solution and 7 *Stereocaulon* extracts were accurately taken as much as 5 µl each and applied to HPTLC plates (in triplicates) using CAMAG Nanomat 4. They were developed at room temperature in a flat bottom chamber (20x20 cm) with solvent system toluene:EtOAc:formic acid (139:83:8). After development, HPTLC plates were dried at 105ºC and scanned densitometrically at 254 nm.

2.7 Antibacterial assay

Antibacterial assays have been carried out on four pathogenic bacteria, which are *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 12228, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. The method used in this assays is a modified microdilution method[21,22] along with p-

iodonitrophenyltetrazolium violet (INT, Merck) as an indicator of bacterial cell viability[23]. Each extract was dissolved with DMSO (10% final volume) and diluted with Mueller Hinton Broth (OXOID) media to a final concentration of 10; 5; 2.5; 1.25; 0.625 and 0.3125 mg / ml. Meanwhile, the isolated compound and chloramphenicol (as a positive control, Merck) were prepared at the final concentration of 5; 2.5; 1.25; 0.625; 0.3125 and 0.15625 mg / ml. A total of 100 µl of each extract concentration and pure isolate were tripled in a microplate well, as well as sterility control (media + DMSO) and growth control (media + DMSO + bacteria). Each microplate well was inoculated with 5 µl bacterial suspension (106 cfu / ml) and incubated at 36 ° C for 18 hours, followed by the addition of 20 µl p-iodonitrotetrazolium (INT) in distilled water (0.5 mg/ml) and reincubated for another 30 minutes. INT is a compound that easily reducted by the presence of the enzyme dehydrogenase in bacteria becoming formazan, which can give a purple color. The change in color from yellow to purple indicates that there are still bacteria in the well.

3 *RESULTS AND DISCUSSION*

3.1 Physical and Spectroscopic Data of Isolation compounds

Atranorin (**1**); colorless needles (EtOAc); mp 196-197˚C; (undepressed in admixture with reference compound) IR νmax (cm−1): 2933, 2360, 1654; UV (Acetonitrile) λmax (log ε): 215 nm (4.2), 251 nm (4.4); ^{1}H NMR (500 MHz, DMSO-*d6*) δH(ppm) 2.03 (s, 3H Me-8'), 2.34 (s, 3H,CH_3-9'), 2.39(s, 3H, CH_3-9), 3.89 (s, 3H,COOMe), 6.42 (s, 1H, H-5'), 6.64 (s, 1H, H-5), 10.21 (s, 1H, -CHO) and 10.52 (s, 1H, OH);^{13}C NMR (125 MHz, DMSO-*d6*): δC(ppm) 107.95 (C-1), 163.54 (C-2), 109.08 (C-3), 161.61 (C-4), 115.78 (C-5), 151.45 (C-6), 164.66 (C-7), 194.03(C-8), 21.27 (C-9), 116.39 (C-1'), 157.43 (C-2'), 110.83 (C-3'), 149.13(C-4'), 115.79 (C-5'), 136.63 (C-6'), 169.83 (C-7'), 21.16 (C-8'), 9.43 (C-9'). Rf 0.86 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

Methyl-β-orcinol carboxylate, MOC (**2**); colorless needles ($CHCl_3$); mp 140-141˚C; (undepressed in admixture with reference compound); IR νmax (cm−1): 3399, 2927, 1728; UV (MeOH) λmax (log ε): 219 nm (3.9), 269 nm(4.15), 303 nm (3.7); ^{1}H NMR (500 MHz, $CDCl_3$) δH(ppm) 2.16 (s, 3H, CH_3-9), 2.45 (s, 3H, CH_3-8), 3.94 (s, 3H, -OCH_3), 6.12 (s, 1H, H-5), 12.13 (s, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$) δC(ppm) 105.33 (C-1), 158.06 (C-2), 108.54 (C-3), 163.24 (C-4), 110.60 (C-5), 140.24 (C-6), 172.69 (C-7), 7.73 (C-8), 24.20 (C-9), 51.93 (OCH_3-10). Rf 0.69 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

Stictic acid (**3**); white amorphous solid; (undepressed in admixture with reference compound); IR νmax (cm−1): 3421.47, 2906.22, 1918, 1692.95; UV (MeOH) λmax (log ε): 242.8 nm (3.85) and 309.4 nm (3.27); ^{1}H NMR (500 MHz, DMSO-*d6*) δH (ppm) 2.18 (s, 3H, CH_3-8'), 2,48 (s, 1H, CH_3-9) 3,89 (s, 3H, -OCH_3-4), 6.60 (s, 1H, H-9'), 7.10 (s, 1H, H-5), 8.27 (s, 1H, OH), 10,44 (s, 1H, CHO-8); ^{13}C NMR (125 MHz, DMSO-*d6*) δC(ppm) 113.25 (C-1), 163.34 (C-2), 114.53 (C-3), 162.65 (C-4), 112.97 (C-5), 151.18 (C-6), 161.00 (C-7), 186.93 (C-8), 21.72 (C-9), 109.30 (C-1'), 152.00(-), 129.43 (C-3'), 148.10 (C-4'), 137.66 (C-5'), 135.91 (C-6'), 166.52 (C-7'), 9.83 (C-8'), 95.41 (C-9'), 56.95 (OCH_3-4). Rf 0.30 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

Lobaric acid (**4**); colorless needles ($CHCl_3$); mp 196-197^{0} C; (undepressed in admixture with reference compound); IR νmax (cm−1): 3164.4, 2960.5, 1720.11, 1662.45; UV (MeOH) λmax (log ε): 213 nm (4.00), 262 nm (3.33) and 293 nm (4.2); ^{1}H NMR (500 MHz, DMSO-*d6*) δH(ppm) 0.87 (t, 6H, CH_3-5", CH_3-5"'), 1.31 (m, 4H, CH_2-4", CH_2-4"'), 1.33 (m, 2H, CH_2-3"'), 1.54 (m, 2H, CH_2-2"'), 2.83 (t, J=7.25, 2H,CH_2-2"), 2.86 (t, J=1.00, 2H, CH_2-1"'), 3.89 (s, 3H, OCH_3), 6.98 (s, 1H, H-3'), 6.93 (d, J=2.4, 1H, H-3), 7.03 (d, J=2.4, 1H, H-5); ^{13}C NMR (125 MHz, DMSO-*d6*) δC(ppm) 111.64 (C-1), 162.80 (C-2), 105.83 (C-3), 164.19 (C-4), 111.06 (C-5), 148.58 (C-6), 152.69 (C-7), 203.31 (C-1''), 39.66 (C-2''), 31.34 (C-3''), 21.54 (C-4''), 13.93 (C-5"), 111.64 (C-1'), 162.15 (C-2'), 106.24 (C-3'), 144.33 (C-4'), 140.64 (C-5'), 133.99 (C-

6'), 168.27 (C-7'), 27.30 (C-1‘''), 30.47 (C-2'''), 31.34 (C-3'''), 21.99 (C-4'''), 13.92 (C-5'''), 56.64 (OCH_3-4). Rf 0.56 with toluene-EtOAc-formic acid (70:25:5) (eluent G).

3.2 Phytochemical investigation and HPTLC (High-Performance Thin Layer Chromatography) metabolite profiling

In this study, five species were collected from six different places are divided into seven samples (Table 1). *S. halei* (1 and 2) and *S. massartianum* (1 and 2) were collected in different locations and altitudes. *Stereocaulon* is a fruticose-type lichen that lives in high altitudes 1200 m ASL with low temperature and is usually found growing on the rock surface exposed by sunlight[24].

Table 1. Location and habitat of 5 Stereocaulon species collected.

Species	No. collection	Coordinate	Altitude	Locality	Habitat
S. montagneanum Lamb	Mon_ FIS-8	0°55'21.1"S 100°47'56.6"E	1300 m	Sirukam, Solok West Sumatra	on the rock, highland
S. halei Lamb (1)	Hal_ FS-13	0°23'24.0"S 100°19'50.9"E	2700 m	Mount Singgalang, West Sumatra	on the rock, slope mountain
S. halei Lamb (2)	FBS-006	0°21'31.5"S 100°17'36.6"E	1350 m	Malalak, West Sumatra	on the rock, highland
S. massartianum Hue (1)	SDA_ Fis-015/DA	1°05'59.4"S 100°45'34.1"E	1400 m	Danau di Atas, West Sumatra	on the rock, highland
S. massartianum Hue (2)	GN7_Fis-016/G7	1°42'06.0"S 101°24'41.0"E	2005 m	Danau Gunung Tujuh, Jambi	on the rock, mountain
S. verruculigerum Hue var. subfurfurascens Lamb	FTS-004	0°58'41.9"S 100°40'45.8"E	1300 m	Mount Talang, West Sumatra	on the rock, lower mountain forest
S. graminosum Schaer	Gra_FS-14	0°23'24.0"S 100°19'50.9"E	2700 m	Mount Singgalang, West Sumatra	on the rock, slope mountain

Compound **1**, **2**, **3**, and **4** (Figure 1). As marker compounds were isolated from *S. halei* (collected from Mount Singgalang) and *S. montagneanum* (collected from Sirukam, Solok) based on previous work[17,18]. The physicochemical properties and all spectral data from isolated compounds were suitable with those previously reported in the literature. (SM S1-S8)

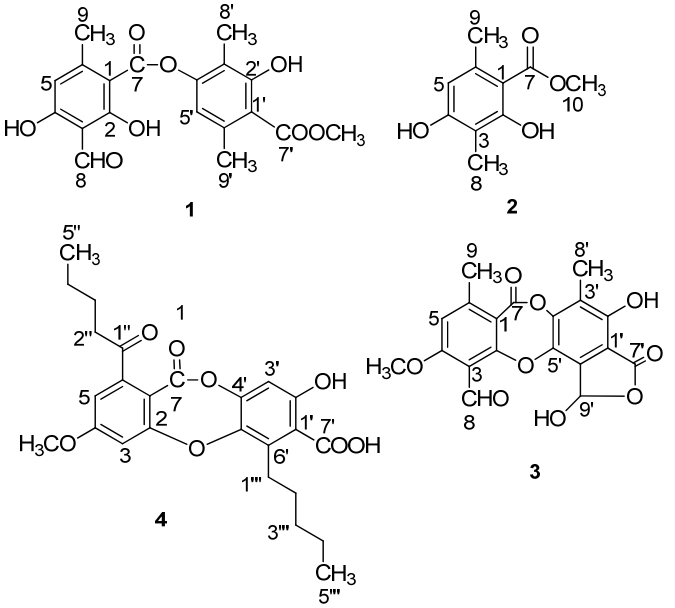
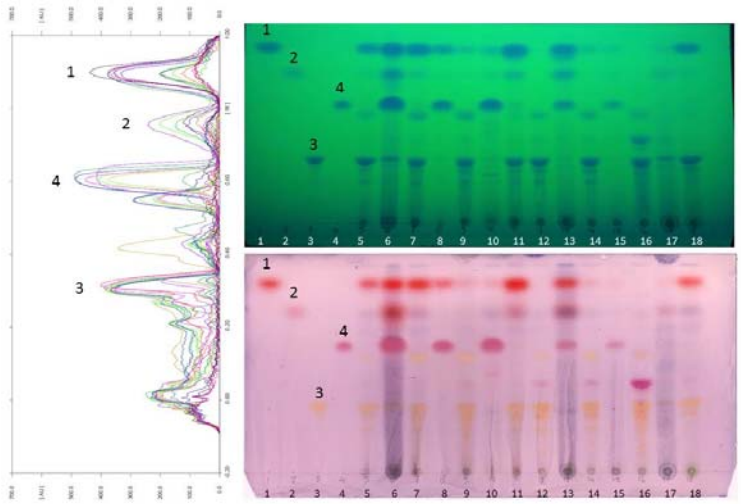


Figure 1. Isolated compounds from *S. halei* and *S. montagneanum*

HPTLC-densitometry metabolite profiling was carried out on different extracts of seven species of *Stereocaulon*. The extraction process was done sequentially by increasing polarity ranging from semipolar to polar (ethyl acetate, acetone, and methanol) to avoid decomposition or degradation of a depside as a major constituent of the genus *Stereocaulon*[25,26]. Identification of the compounds was realized for four major compounds (atranorin, MOC, lobaric acid, and stictic acid), and this study focused on detecting the similar compounds between the five species. Atranorin (track1; Rf = 0.80, λmax 220, 254 and 280 nm) was detected in all five species of *Stereocaulon* especially in EtOAc extracts (Table 2) (Figure 2 and SM Figure 9-11). Whereas in all acetone extracts, the atranorin spot began to thin out (except for *S.halei* (1) and *S.graminosum*, track 13 and 18 respectively, still look strong) even in methanol extracts the spot were not visible at all (Figure 3 and SM Figure 12-14). Atranorin, a β-orcinol depside, is the most common and widely found compound in almost all *Stereocaulon* species[9,10]. MOC (track 2; Rf = 0.69, λmax 220, 254, and 280 nm). It is only seen in EtOAc and MeOH extracts, so it is estimated that this monoaromatic compound is a precursor to depside biogenesis[27-29] and the result of atranorin depside degradation undergoes alcoholysis[30,31]. Lobaric acid (track 4; Rf = 0.56, λmax 220, 254 and 280 nm) was seen in both *S. halei* 1 and 2 (track 6, 8, 13 and 15) and *S. verruculigerum* (track 10) in EtOAc and acetone extracts. Furthermore, *S. montagneanum* (track 5 and 12), *S. massartianum* 1 and 2 (track 7, 9, 14 and 16), and *S. graminosum* (track 11 and 18) were detected to contain stictic acid (track 3; Rf = 0.30, λmax 220, 254 and 280 nm) in EtOAc and acetone extracts, respectively. Man-Rong described three chemotypes that have been found on *S. massartianum*[32]: the first one contains stictic and norstictic acids, the second one lobaric acid only, and the third one lobaric, stictic and norstictic acids. Lobaric and stictic acids are prenyl and furano-type depsidones, respectively, commonly discovered in the *Stereocaulon* genus[1]. Three types of prenyl-depsidones have been reported, namely lobaric acid, colensoic acid, and norlobaric acid, which were isolated from *S. colensoi* and *S. paschale*[33,34]. Stictic acid has been found in many lichen families in the Parmeliaceae, Pertusariaceae, and Usneaceae families[18,35].

As previously reported, generally, stictic acid in lichen experiences a chemical phenomenon known as stictic acid chemosindrome. This phenomenon shows that stictic acid can experience various degrees of oxidation of functional groups such as CHO, COOH, CH_2OH or OH and methylation of phenolic groups such as OCH_3, OH[18,36,37].



1= atranorin
2= MOC
3= Stictic acid
4= lobaric acid
19= MeOH extr. of *S. montagneanum*
20= MeOH extr. of S. halei (1)
21= MeOH extr. of *S. massartianum (1)*
22= MeOH extr. of *S. halei (2)*
23= MeOH extr. of *S. massartianum (2)*
24= MeOH extr. of *S. verruculigerum*
25= MeOH extr. of *S. graminosum*

Figure 2. The densitogram of marker compounds and extracts (EtOAc and Acetone) of genus *Stereocaulon* (left) scanned at 245 nm. The HPTLC profile in UV light at 254 nm developed with toluene:EtOAc:formic acid (139:83:8) (top right) and visualized with anisaldehyde sulfuric acid (bottom right)

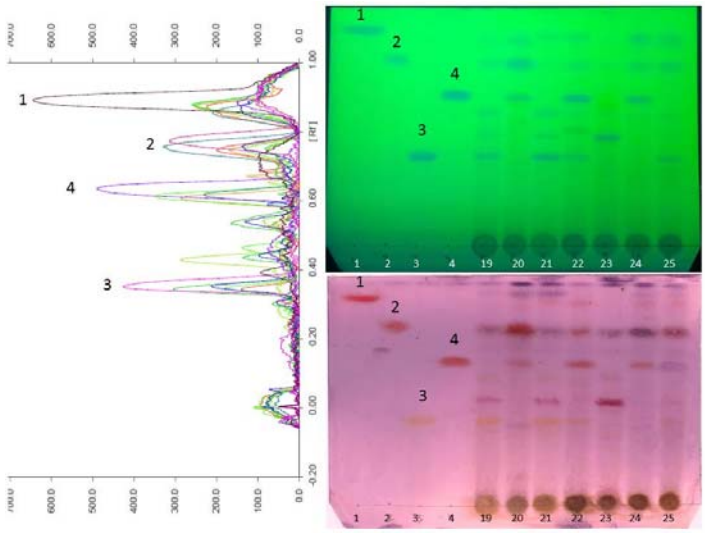


Figure 3. The densitogram of marker compounds and MeOH extracts of genus *Stereocaulon* (left) scanned at 245 nm. The HPTLC profile in UV light at 254 nm developed with toluene:EtOAc:formic acid (139:83:8) (top right) and visualized with anisaldehyde sulfuric acid (bottom right)

Table 2. Metabolites detected with HPTLC-UV in genus *Stereocaulon*

no	R_f	λmax (nm)	Compounds				Extracts of *Stereocaulon* species						
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	0.11	280, 310					+		+		+		+
2	0.20	220, 254					+		+		+	+	+
3	0.24											+	
4	0.30	220, 254, 280			+		+	-	+		+	-	+
5	0.39	254, 280, 320						+					
6	0.40	280, 320								+			
7	0.45											+	
8	0.49	220, 254							+		+		
9	0.51	220, 254					+					+	+
10	0.56	220, 254, 280				+		+	-	+			+
11	0.66	320					+						
12	0.69	220, 254, 280		+			+	+	+	-	-	+	+
13	0.80	220, 254, 280	+				+	+	+	+	+	+	+
			1	**2**	**3**	**4**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
1	0.08	280, 310					+						
2	0.11	220, 254							+				
3	0.20	220, 254					+						
4	0.27	220, 254						+	+			+	+
5	0.30	220, 254, 280			+		+	-			+	+	+
6	0.39	220, 254					+	+		+	+	+	
7	0.40	220, 254							+				+
8	0,49	220, 254					+		+		+	+	+
9	0.54	220, 254, 280						+		+			
10	0.56	220, 254				+						+	+
11	0.69	220, 254, 280		+			-	+	-	-	-	+	+
12	0.77	220, 254, 280						+					
13	0.80	220, 254, 280	+						+	+	+	+	+
			1	**2**	**3**	**4**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
1	0.30	220, 254			+		+		+				+
2	0.32	220, 254					+		+	+		+	+
3	0.38	220, 254, 280									+		
4	0.40	220, 254							+				
5	0.42	220, 254					+			+			+
6	0.51	220, 254					+		+	+	+		+
7	0.56	220, 254, 280				+							
8	0.58	220, 254					+	+		+			

9	0.64	220, 254									+	+		
10	0.69	220, 254		+			+	+	-	+	+	+	+	
11	0.80	220, 254, 280, 320, 365	+				+	+	+	+		+	+	

1 = atranorin, **2**= MOC, **3** = stictic acid, **4** = lobaric acid, **5**= EtOAc extr. of *S. montagneanum,* **6**= EtOAc extr. of *S. halei* (1), **7**= EtOAc extr. of *S. massartianum* (1), **8**= EtOAc extr. of *S. halei* (2), **9**= EtOAc extr. of *S. massartianum* (2), **10**= EtOAc extr. of *S. verruculigerum,* **11**= EtOAc extr. of *S. graminosum,* **12**= Acetone extr. of *S. montagneanum,* **13**= Acetone extr. of *S. halei* (1), **14**= Acetone extr. of *S. massartianum* (1), **15**= Acetone extr. of *S. halei* (2), **16**= Acetone extr. of *S. massartianum* (2), **17**= Acetone extr. of *S. verruculigerum,* **18**= Acetone extr. of *S. graminosum,* **19**= MeOH extr. of *S. montagneanum,* **20**= MeOH extr. of *S. halei* (1), **21**= MeOH extr. of *S. massartianum* (1), **22**= MeOH extr. of *S. halei* (2), **23**= MeOH extr. of *S. massartianum* (2), **24**= MeOH extr. of *S. verruculigerum* **25**= MeOH extr. of *S. graminosum*

3.3 Quantification of atranorin (1) in 7 extracts genus Stereocaulon using HPTLC

To date, there have been no reports of atranorin (**1**) quantification from the extracts of the *Stereocaulon* genus. In this measurement, we used a standard lichen eluent (toluene:EtOAc:formic acid (139:83:8))[20] with an atranorin Rf value of 0.87 detected at a wavelength of 254 nm. Based on the correlation between the concentration of atranorin standard (x) and peak area (y), the equation y = 34.167x + 1270.5 (r = 0.9963)(SM figure 15) was obtained. The highest atranorin content was detected from *S. graminosum* with atranorin value 325,498 mg/g extract and equivalent to 0.065 mg/g dried whole thallus while the lowest was from *S. verruculigerum* with 23,356 mg/g extract and 0.023 mg/g dried whole thallus (Table 3).

This quantification process is needed to determine specifically the content of atranorin in *Stereocaulon* and the correlation with its species and habitat. This is important due to atranorin is not only found in the genus *Stereocaulon* but generally is found in most lichen groups such as Cladoniaceae, Lecanoraceae, Parmeliaceae dan Stereocaulaceae[38]. Furthermore, atranorin is reported to have various pharmacological activities including anti-inflammatory[39], wound healing agent[40], antioxidant[35], and sunscreen substances[41].

Table 3. Calculation of atranorin concentration from extracts and dried whole thallus genus Stereocaulon

No	**Species**	**total extract (g)**	**Atranorin concentration**	
			per 1 gram extract (mg)	**per 1 gram dried-whole thallus (mg)**
1	*S. montagneanum* Lamb	2.118	176.174	0.035
2	*S. halei* Lamb (1)	2.706	270.534	0.054
3	*S. halei* Lamb (2)	2.647	94.974	0.019
4	*S. massartianum* Hue (1)	1.220	165.109	0.033
5	*S. massartianum* Hue (2)	1.233	32.002	0.032
6	*S. verruculigerum* Hue *var. subfurfurascens* Lamb	0.798	23.356	0.023
7	*S. graminosum* Schaer	1.029	325.498	0.065
Conc. 10mg/10 ml				

3.4 Antibacterial activity

Antibacterial test of pure extracts and isolates from the genus *Stereocaulon* lichen has been carried out using the liquid microdilution method, by determining the Minimum Inhibition Concentration (MIC) of extracts and pure isolates. This method was chosen because the samples and reagents used are relatively small but have a high level of sensitivity and provide quantitative results[21,22]. The test results showed all extracts in the genus *Stereocaulon* lichen had antibacterial activity against test bacteria but had different MIC values. Ethyl acetate extracts from *S. halei*, *S. massartianum*, and *S. graminosum* and acetone extract from *S. massartianum* showed the highest antibacterial activity against test bacteria with a range of MIC = 0.1-0.6 mg/mL. Furthermore, from the four isolated compounds tested, lobaric acid and MOC showed potential antibacterial activity against the four test bacteria with MIC values = 1.5-3 mg/mL.

The results of this test were in line with several other studies that reported a good potential of the genus *Stereocaulon* extract against pathogenic bacteria. Crude acetone extract of *S. massartianum* collected in the Philippines provides a large diameter of inhibition (19 mm) against *S. aureus* and *B. subtilis*[42]. Other studies show that an ethanolic extract from *S. foliolosum* inhibits the growth of M. tuberculosis H37Rv with MIC 500 mg/mL which is compared to rifampicin and isoniazid (MIC value 0.25 and 0.1 mg/mL, respectively)[43].

Table 4. Antibacterial activity from extracts and isolated compounds of genus *Stereocaulon*

Test materials	**MIC (mg/mL)**			
	SA	**PA**	**EC**	**EF**
S. montagneanum				
EtOAc extr.	10	5	2.5	2.5
Acetone extr.	5	2.5	2.5	5
MeOH extr.	10	2.5	5	5
***S. halei* (1)**				
EtOAc extr.	2.5	0.3	0.6	0.6
Acetone extr.	5	5	10	2.5
MeOH extr.	2.5	2.5	10	5
***S. massartianum* (1)**				
EtOAc extr.	2.5	2.5	5	0.1
Acetone extr.	2.5	2.5	5	0.1
MeOH extr.	2.5	5	5	5
***S. halei* (2)**				
EtOAc extr.	2.5	5	5	5
Acetone extr.	5	5	10	2.5
MeOH extr.	2.5	2.5	10	5
***S. massartianum* (2)**				
EtOAc extr.	2.5	2.5	5	2.5
Acetone extr.	2.5	2.5	5	2.5
MeOH extr.	2.5	5	5	5
S. verruculigerum				
EtOAc extr.	2.5	5	5	5
Acetone extr.	5	2.5	10	2.5
MeOH extr.	5	2.5	5	5
S. graminosum				

EtOAc extr.	0.6	0.6	0.6	0.6
Acetone extr.	1.2	2.5	2.5	0.6
MeOH extr.	2.5	2.5	2.5	0.3
Atranorin (1)	12	6	6	6
MOC (2)	3	3	3	1.5
Stictic acid (3)	-	-	-	-
Lobaric Acid (4)	3	1.5	1.5	1.5
Chloramphenicol	0.6	0.3	0.6	0.6

All values presented are Mean ± SD of triplicate readings

4 CONCLUSIONS

Metabolite profiling from 5 species showed that there was an atranorin compound in all test samples while stictic acid and lobaric acid were not. This appears that atranorin can be a marker compound for the genus *Stereocaulon*. The content of atranorin in each species also varies and is influenced by habitat and growing conditions. Based on the results of the quantification test, *S. graminosum* has the highest content of all samples. The result of two samples from the same species but of different habitats showed significant differences as well. This could be seen in the *S. halei* samples collected from a place with an altitude of 2700 m having more atranorin content than those growing at an altitude of 1350 m.

Furthermore, antibacterial tests on 4 pathogenic bacteria showed that all extract inhibited the growth of the tested bacteria. The ethyl acetate extract of *S. halei, S. massartianum, S. graminosum*, and acetone extract from *S. massartianum* have the highest antibacterial activity with a range of MIC = 0.1-0.6 mg/mL. These three species also exhibit stronger and specific antibacterial activity against *E. feacalis*. On the other hand, isolated compounds such as lobaric acid and MOC have MIC values = 1.5-3 mg/mL, whereas atranorin itself does not show satisfactory values compared with chloramphenicol.

ACKNOWLEDGEMENTS

We are grateful to the Fundamental Grant 2019 DRPM-DIKTI – The Ministry Research, Technology, and Higher Education of the Republic of Indonesia to FI (contract No. 051/SP2H/LT/DRPM/2019). Dr Harrie J. M. Sipman, Botanischer Garten und Botanisches Museum Berlin-Dahlem, Freie Universitat Berlin, is acknowledged for the identification of the lichen. We also thank Dr. Ahmad Darmawan and Sofa Fajriah, Pusat Penelitian Kimia, LIPI, Serpong, for the collection of NMR data.

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التوصيف الكيميائي النباتي والأنشطة المضادة للبكتيريا للخلاصات من خمسة أنواع من حزاز سومطرة من جنس ستيروكولون *Stereocaulon*

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ملخص

في الجرد والتحقيق المستمر في حزاز سومطرة، تم جمع خمسة أنواع من جنس ستيروكولون. من خلال تحليل التوصيف الأيضي، تم عزل المركبات الرئيسية الأربعة الموجودة في هذه الأنواع (مثل الأترانورين، ميثيل–β – أورسينول كربوكسيلات (**MOC**)، حمض اللوباريك، وحمض الستيكتيك) بسرعة كمركبات علامة باستخدام طرق كروماتوجرافيا تم تطويرها لـ س. حالي **S. halei** و س. مونتاجنيوم ***S. montagneanum***. تم تحليل الخلاصات من كل نوع باستخدام كروماتوجرافيا الطبقة الرقيقة عالية الأداء - (**HPTLC**) قياس الكثافة بخمسة أطوال موجية (220، 254، 280، 320، و 365 نانومتر). اترانورين و دوران الانقلاب الطولي **MOC** هما مركبان موجودان في جميع أنواع ستيروكولون الخمسة المختبرة. بعد ذلك، تم إجراء تقدير كمية الأترانورين عن طريق مسح قياس الكثافة عند 254 نانومتر من خلاصة أسيتات الإيثيل لكل نوع من أنواع ستيروكولون. تم الكشف عن أعلى تركيز للأترانورين في س. جراميوسوم ***S. graminosum*** (325.498 مجم / جم خلاصة) ما يعادل 0.065 مجم / جرام من الثاليوس الكامل المجفف، بينما كان أقل تركيز من س. فيروكوليجيروم ***S. verruculigerum*** (23.356 مجم / جرام خلاصة) و 0.023 مجم / جرام مجفف كامل ثالوس. إضافة إلى ذلك، تم تقييم جميع الخلاصات والمركبات الرئيسية الناتجة عن العزلة من حيث نشاطها المضاد للبكتيريا باستخدام طريقة التخفيف الدقيق. أظهرت خلاصات إيثيل أسيتات والأسيتون من س. ماسارتيانوم ***S. massartianum*** أعلى نشاط مضاد للجراثيم ضد هـ فيساليس **E. faecalis** (مكس **MIC** = 0.3125 مجم / جرام).

الكلمات الدالة: اترانورين • قياس كثافة–**HPTLC** • حزاز • تحديد خصائص المستقلبات • ستريكولون ***Stereocaulon*** .

تاريخ استلام البحث 2020/4/2 وتاريخ قبوله للنشر 2020/5/20.

Jordan Journal of Pharmaceutical Sciences, Volume 14, No. 2, 2021

Clinical microbiology laboratory isolates: prevalence and gender variation

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ABSTRACT

Data archives of clinical microbiology laboratory (CML) are a rich source of valuable information. In this study, CML data from two tertiary care hospitals in Jordan were reviewed, in order to identify the source prevalence of microorganisms and the dissemination of Extended Spectrum Beta-Lactamase (ESBL) bacteria. The association between gender and the microorganism or specimen source was investigated. Data collected from each hospital was screened and filtered according to predetermined exclusion criteria. Of 20726 records screened, 5547 valid specimens with positive growth were considered. From these specimens 7143 isolates were identified to genus or species level. The most common specimens were identified and their association with gender was statistically determined and discussed. The prevalence of microorganisms in different specimens was compared with the literature, and variation from the expected results was explained. In addition, some associations were observed between gender and the predominating microorganisms. High dissemination rate of ESBL-producing bacteria was observed among Gram-negative bacteria (57%), indicating serious challenges facing clinicians in finding suitable treatments. Reviewing CML data can aid in the study of trends in the dissemination of microorganisms in specific population groups and can aid clinicians in amending their treatment protocols.

Keywords: Enterococcus, Streptococcus agalactiae, ESBL, gender, prevalence, laboratory, urine culture, vaginal swab.

1. INTRODUCTION

Infectious diseases are known to cause a large burden on the health sector and economy of many countries. They are responsible for a substantial proportion of morbidities and mortalities among the population. The causative agents of these infections vary from bacterial and viral infections to fungal and parasitic ones. In order to treat or manage these illnesses, diagnosis is a crucial step in order to identify the pathogen and to commence a suitable treatment protocol. Diagnosis involves many aspects including clinical manifestation, physical examination, medical history, travel history, imaging and an important confirmative tool, laboratory testing [1]. Although some infections can be identified clinically (ie: the so-called "frank" pathogen), others require clinical microbiology laboratory to identify the causative agent [1]. Besides identifying the etiologic agent of the disease, clinical microbiology laboratories provide *in vitro* information about the susceptibility of the isolated microorganisms towards antimicrobial drugs. The retrospective review of microbiology laboratory data can be a valuable tool in estimating the prevalence of certain microorganisms among specific population groups or under certain conditions, and also helps in tracing the trends in the microorganism's dissemination throughout a host population [2–4].

Variation in gender could predispose a sub-section of a

Received on 12/7/2020 and Accepted for Publication on 30/4/2021.

host population to some specific infectious diseases more than the opposite gender. Due to anatomical and physiological differences between males and females, the microbiota in certain areas in the body vary and consequently, the incidence of some infections will vary between the two genders [5]. Some studies have shown that patient's gender could be a risk factor for some microbial infections [5,6]. Furthermore, the WHO has issued a report indicating the effect of sex and gender on population health in emerging infectious diseases and epidemic-prone diseases [7]. In this report, biological differences between males and females, and the physiological changes women undergo during pregnancy are observed to have an effect on the immune response and hence, upon overall vulnerability and susceptibility towards infections and subsequent disease. Similarly, the report also made reference to observed gender differences in physical activity patterns, which could lead to different levels of exposure towards different infectious agents and gender differences in accessibility to economic resources, which could act as a barrier to access effective health care. Such differences will undoubtedly result in gender variations in responses to emerging infectious diseases [7].

Over several years, bacteria have developed mechanisms to resist antibiotics. In fact, antimicrobial resistance has become a public health crisis with the emergence of multiple drug resistance (MDR) in several clinically important species of bacteria. This crisis is taking the world into what is called the *"post antibiotic era"*, where previously minor infections might no longer be curable and may result in fatality due to lack of effective antibiotics [8]. Among these MDR microorganisms are the Extended Spectrum Beta Lactamase producing bacteria. These bacteria produce a group of enzymes called Extended Spectrum Beta Lactamases (ESBLs). ESBLs confer resistance to a broad range of β-lactams, such as penicillins and first-, second- and third-generation cephalosporins; and azotreonam [9]. These enzymes are primarily produced by Gram-negative bacteria, mainly Enterobacteriaceae, *Acinetobacter*, and *Pseudomonas aeruginosa* [9]. Recent studies indicated that the spectrum of antibiotic resistance of the ESBL-producing bacteria is expanding to include multiple antibiotic classes, leaving the clinician with very limited treatment options and the patient with poorer prospective outcomes [10,11].

The aim of this study was to explore clinical microbiology laboratory results in two tertiary care hospitals in Jordan during 2017 and 2018, in order to identify the most common specimens provided, the predominating microorganisms in different specimens and the prevalence of ESBL-producing bacteria in these clinical specimens. In addition, the possibility of gender-association with the specimens and microorganisms was also investigated. It is proposed that such information will inform our knowledge of the distribution and host-susceptibility of different microorganisms among the general population, in addition to the extent of dissemination of ESBL-producers.

2. Methodology

This is a retrospective study, in which the microbiology laboratory results from two hospitals in Amman, Jordan were evaluated for the years 2017 and 2018. The first hospital is a public sector academic tertiary care hospital with 600 beds. On an annual basis, this hospital receives more than 600,000 patients in the outpatient clinics and more than 40,000 patients are admitted for treatment. The second hospital is a private sector tertiary care hospital with 160 beds. On an annual basis this hospital deals with about 100,000 patients as outpatients and in-patients.

The study received an ethical approval from the Ethics Committee of Scientific Research, Ministry of Health, Jordan (code: MOH REC 1900018). All necessary approvals were also obtained from the administration of each hospital. The clinical laboratories of the two hospitals relied on Vitek®-2 systems (bioMérieux, France) for microbial identification. The laboratory data was retrieved from the servers of the microbiology laboratory with the help of the hospital's information technology departments.

The following information was obtained: Patient's file number (a unique number of each patient), age, gender, sample date, sample source, microbial culture result (microorganism type and whether it is ESBL-producer or non-ESBL producer).

The data collected were obtained as raw data and screened based upon exclusion criteria. The criteria included the exclusion of any duplication in data entry (e.g same patient's file number on the same date and same sample), invalid data entry or missing information, and any specimen showing no microbial growth or the result was reported as *"no significant growth"* or *"normal flora"*. Moreover, whenever the same microorganism was isolated from a specimen that was cultured within three weeks of the previous culture it was excluded, because it was assumed that the microorganism isolated in the second specimen is the same causative agent of the first culture. Hence, three weeks were considered a suitable cut-off in most treatment guidelines, since the majority of infections are expected to resolve with treatment within 14 days [12,13].

2.1 Statistical analysis

Statistical analysis of the data was performed using SPSS version 20.0 (SPSS Inc., Chicago, IL). Descriptive statistics was used to describe the specimens tested. Categorical variables were presented as valid percentages. Non parametric Chi square test was used to test for association between the categorical variables. A *P*-value of < 0.05 was considered significant

3. Results and discussion

The total number of raw laboratory results screened from in-patients and out-patients from the two hospitals was 20726; 14498 from the first hospital and 6228 from the second hospital. After filtering the data according to the exclusion criteria, the total number of specimens with valid laboratory results and significant microbial growth was 5547; 3775 and 1772 from the first and second hospitals respectively. For these specimens, the age of the patients ranged from 3 days to 102 years. From the specimens reported, 7143 microorganisms were isolated and identified to the genus or species level, except for 146 isolates which were reported as coliforms (Table 1). Gram-positive bacteria were the predominant group of isolated microorganisms (52%) followed by Gram-negative bacteria (45.3%), and then yeast and fungi (2.7%). The most common Gram-positive bacteria isolated were Enterococci (18.8%), followed by Staphylococci (17.5%) and Streptococci (15.7%). The majority of Streptococci belonged to Group B (*S. agalactiae*) which constitutes 95.1% of all Streptococci. For Gram-negative bacteria, the Enterobacteriaceae family was the predominating one, where the most common bacteria isolated were *Escherichia coli* (32.1%), followed by *Klebsiella* (8.6%).

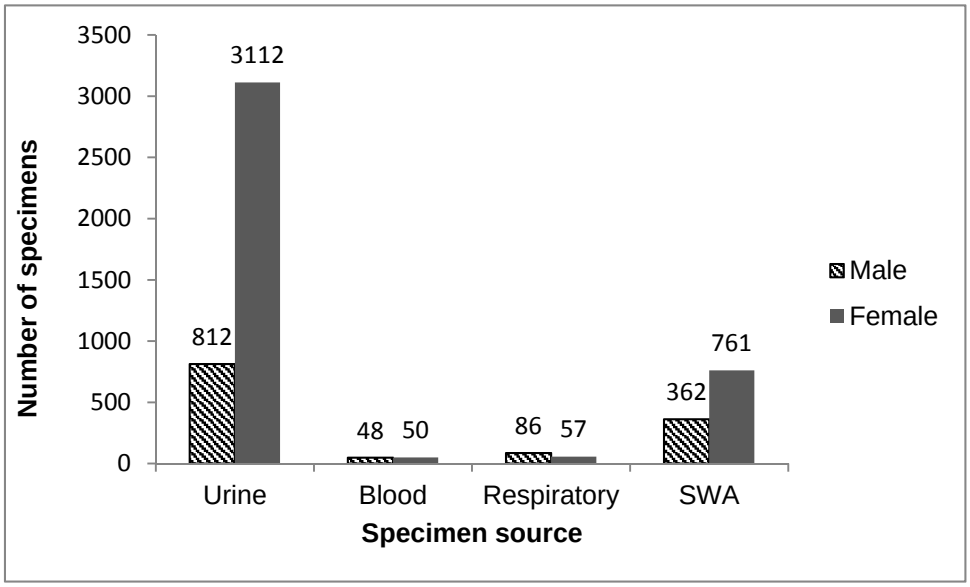

Fig. 1. Numbers of specimens identified between males and females based on specimen source. SWA: Skin, wound and abscess.

Table 2 shows the distribution of the isolates based on the source of specimens. These isolates were detected in the specimens as pure culture or in combination with other microorganisms. Urine samples were the most commonly infected of the specimens cultured (N=3924), followed by skin, wound and abscess (SWA, N=1123), respiratory (N=143), blood (N=98) and vaginal (N=81) specimens.

3.1 Urine Specimens

Urinary tract infections (UTI) are very common worldwide and considered one of the leading causes of visits to primary care clinics. A global study found that in developed countries, UTIs are the second most common infectious disease, after respiratory tract infections, that cause patients to visit clinics [14]. In developing countries, UTIs are the third most numerous reason for clinical visits, preceded by respiratory tract infections and tuberculosis [14]. UTIs account for a significant proportion of antibiotic prescription and dispensing [15,16]. In Jordan, they were found to be the second most numerous infectious disease, after respiratory tract infections, to which antibiotics were dispensed with or without prescription [16].

We suggest that the reason why urine and SWA specimens exceeded respiratory tract specimens in our study is that almost 80% of clinically diagnosed respiratory infections are caused by viruses such as influenza viruses, parainfluenza viruses, adenovirus and rhinoviruses [17]. In addition, even in non-viral respiratory infections, physicians may rely on physical examination, and clinical symptoms or other diagnostic tools (e.g: x-ray) rather than laboratory tests to determine the appropriate treatment [18]. On the other hand, UTIs are known to occur more frequently in females than in males [19], which was obvious in our results where 3112 positive urine specimens from females were reported compared to 812 urine specimens from males (Figure 1).

It is known that the leading cause of urinary tract infections is *E. coli* [20,21]. It is responsible for 70 to 95% of both upper and lower UTIs. The remainder of UTI infections are caused by *Klebsiella* spp., *Enterococcus faecalis*, *Proteus* spp., *Staphylococcus* spp., other Enterobacteriaceae and yeasts [19,21]. Consistent with

this, several microorganisms were isolated from urine in this study. The most predominant were *E. coli* (51.7%) followed by *Enterococcus* spp. (34%), *Staphylococcus* spp. (30.2%), and then *Klebsiella* spp. (11.8%; Table 2). From the literature it appears that the observed prevalence of the genus *Enterococcus* in urinary tract infections is increasing [22]. It is implicated in urinary tract infections and in asymptomatic bacteriuria. A five year study in a veteran's hospital in the United States identified enterococci in positive culture urine specimens in 22.5 % of the specimens compared to our finding of 34%. The problem with enterococci is their increasing resistance to a broad range of antimicrobials. This has led to the publication of guidelines for the diagnosis and management of UTIs versus asymptomatic bacteriuria caused by enterococci to reduce the overuse of antibiotics [2,3].

The predominating microorganisms identified in urine in our study were also seen in a previous study by Flores-Mireles *et al.* (2015) [19], who found that the prevalence of microorganisms in UTIs varies between complicated and uncomplicated infections. Other than *E. coli*, the top three microorganisms prevailing in uncomplicated UTIs were *K. pneumoniae*, *Staphylococcus saprophyticus* and *Enterococcus* spp. while in complicated UTIs the top three microorganisms excluding *E. coli* were *Enterococcus* spp., *K. pneumoniae* and *Candida* spp.

Interestingly, Gram-positive bacteria, enterococci, staphylococci and streptococci, in relation to other microorganisms exhibited a significantly higher association with female urine specimens than with male urine specimens ($p<0.001$, $p<0.001$, $p=0.043$ respectively). While Gram-negative bacteria (*E. coli* and *Klebsiella* spp.) exhibited a significantly higher association with male urine specimens than with females ($p<0.001$, $p=0.015$). In line with these results, it has been estimated that about 5-25% of uncomplicated urinary tract infections in women are caused by Gram-positive bacteria mainly *S. saprophyticus, Enterococcus faecalis* and Group B Streptococci GBS [23]. These infections are most common in non-pregnant women of child bearing age. Nevertheless, in pregnant women Gram-positive bacteria are more often seen as etiologic agents of UTIs [23]. This was observed in this study where enterococci and staphylococci in urine specimens were significantly more associated with women in the age group 13-50 years than in the other age groups (Pearson's Chi square, $p<0.001$ for both bacteria). However, the presence of streptococci was not significantly different between these age groups.

3.2 Skin, Wound & Abscess Specimens

The second most common specimens in this study were from SWA samples. Specimens tested and reported from females were twice the number of those from males (761 vs 362 specimens, Figure 1). This finding is in agreement with that reported by Shallcross *et al*. (2014). In their study, data collected between 1995 and 2010 in the UK showed that the first time consultation at a general practitioner for a boil or abscess was 512 for females and 387 for males per 100,000 person-years [24].

Bacterial skin infections are mainly caused by staphylococci and streptococci [25]. In this study streptococci comprised the highest proportion of SWA specimens (69.8%), while staphylococci comprised only 3.3% (Table 2). This doesn't necessarily mean that staphylococci play a minor role in these infections. Rather, it could imply that these infections were treated empirically with antibiotics and those not responding were referred to the laboratories to help in diagnosis and treatment regime. As mentioned earlier the vast majority of streptococci isolated in this study were GBS. GBS are known to colonize the human genital and gastrointestinal tracts and to a lesser extent the respiratory tract [26]. This group is a leading cause of morbidity and mortality in neonates and pregnant women [26]. In newborns GBS are responsible for many diseases (such as bacteremia, sepsis, pneumonia, and meningitis), all of which can be fatal in infants. Furthermore, invasive GBS diseases are

increasingly seen in non-pregnant adults, especially among the elderly and people with underlying medical conditions [27]. Of the invasive GBS cases in non-pregnant adults, skin and soft tissue infections are among the most frequently reported cases [27]. This is consistent with our findings, where GBS in SWA infections is significantly higher in the age group >75 years than the other age groups (Pearson's Chi square, p=0.008).

A remarkable proportion of SWA specimens harbored Gram-negative bacteria (~20%). Skin infections with Gram-negative bacteria occur mainly in diabetic patients or the immunocompromised [25]. In fact diabetes is wide spread among Jordanians and in 2016 it was estimated that 46% of Jordanians who are above 25 years are diabetic, whether diagnosed or not [28], which could explain the high occurrence of Gram-negative bacteria in these specimens.

Other than bacteria as a cause of skin and soft tissue infections, fungal infections are on the rise [29]. In these fungal infections, cutaneous and soft tissue infections are usually caused by *Candida* spp. [30,31]. The role of *Candida* spp. can be detected in our results where *Candida* was isolated from 5.2% of the SWA specimens comprising the fourth most dominant microorganism in these specimens (Table 2). *Candida* spp. was found to be associated with females more than with males (p=0.01). This association has been reported previously in a study by Heidrich *et al.* (2016). In their sixteen-year retrospective study in Brazil, they found that dermatomycoses caused by the genus *Candida* were more predominant in women than in men [32].

3.3 Respiratory Specimens

Respiratory tract specimens were the third most common group in this study. Specimens from males were one and a half times greater in number than those for females (86 versus 57, Figure 1) indicating a higher incidence of respiratory infections among males than among females. One of the reasons for this could be linked to smoking, which is more prevalent among males than females in Jordan. Smoking is considered a major risk factor for respiratory diseases [33]. Jordan, has one of the highest smoking prevalence in the world. Males from different sample groups (eg: health care workers, college students, youths etc.) were found to significantly outnumber females in smoking [34].

It is known that among the most common bacterial cause of respiratory tract infections are streptococci [35]. *S. pneumoniae* is considered the most common cause of community acquired acute bacterial pneumonia [35]. In general, some Streptococci are part of the normal human oral microflora. Some species, such as *Streptococcus pyogenes* and *Streptococcus pneumoniae*, are considered to be occasional respiratory tract residents [36]. In line with this, the microorganism isolated in greatest numbers from the respiratory system specimens in this study were the streptococci. However, the vast majority of the isolated streptococci were GBS, which was unexpected and requires further investigation. Nevertheless, this high prevalence of GBS could imply that their occurrence in infections is increasing as they also comprised the majority of streptococci in SWA infections. One suggestion for this high prevalence of GBS is that these isolates could have high resistance to antimicrobials, which enables them to survive empirical treatment given to patients.

In our results, the second most frequently isolated microorganism from respiratory specimens was *Candida* spp., in 16.8% of specimens (Table 2). *Candida* spp. is part of the normal human flora of the oral cavity and its detection in the respiratory system has often been thought to be the result of re-localisation of this normally commensal microorganism [37]. Unfortunately, there is no test or criteria to define the isolation of *Candida* from respiratory tract specimen as being a contamination, commensalism, colonization or infection. However, in recent years it has been recognized that the interpretation of the detection of *Candida* spp. in the respiratory tract should be based on clinical and microbiological context [37].

3.4 Blood Specimens

In this study *E. coli* and *Klebsiella* spp. were the most predominant bacteria in the blood cultures (34.7% and 30.6% respectively, Table 2). Compared to a multicenter study in Hubei Province, China, between 2014 and 2016, *E. coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* were reported as the most common pathogens responsible for nosocomial blood stream infections [38]. In another study, it has been shown that Gram-negative bacteria, in particular Enterobacteriaceae, were among the main causative agents of bacteremia [39].

Over time, Enterobacteriaceae have become resistant to antibiotics with the emergence of ESBL and carbapenemase producing strains [39,40]. This can be seen in our results where the predominance of ESBL-producing *E. coli* and *Klebsiella* in blood specimens was 33.7% and 28.7% respectively (Table 3), which is very close to the predominance of these two bacteria as both non-ESBL and ESBL-producers (34.7% and 30.6% respectively, Table 2). This indicates that the vast majority of the bacteria isolated from blood specimens are ESBL-producers. These bacteria are highly resistant to many antibiotics and are extremely difficult to eradicate [39,40]. Thus, their associated infections have a high potential to predispose to blood infections due to the invasion of the bacteria into blood, which explains their predominance in blood specimens.

3.5 Vaginal Specimens

The colonization of the vagina is affected partly by estrogen levels, which change with the female's age, menstrual cycle and pregnancy [5]. In some cases commensal microorganisms such as *Gardnerella vaginalis*, *E. coli* and GBS can cause vaginal infections and subsequent disease [41]. In our study the most predominant vaginal isolates were streptococci and *Candida* spp. GBS colonization in the genital tract [26] could explain its predominance in the high vaginal swabs in our study (45%, Table 2). This predominance in the vagina is consistent with a previous study in Jordan, where nearly 20% of specimens collected from the vagina of pregnant women were colonized with GBS [42]. In another study on non-pregnant women in Oregon USA, Leclair *et al*. [37], found that GBS prevalence was 22.8% and that there was a significant relationship between vaginitis and GBS colonization in the vagina.

The second predominating microorganism in high vaginal swabs is *Candida* spp. (32.1%). Although *Candida* can be found normally in the vagina in many women where it exists as a unicellular commensal under normal circumstances, changes in the vaginal environment, such as hormonal change, diabetes, use of oral contraceptives, etc, can encourage the growth of *Candida* in a non-commensal and pathogenic form, thus predisposing to vulvovaginal candidiasis [5]. Vaginal candidiasis is one of the most common fungal vaginal infections [5], which is in agreement with our findings where *Candida* is the most predominant fungus/yeast isolated.

Of the 3236 Gram-negative bacteria isolated, 1843 (57%) were phenotypically identified as ESBL-producers (Table 1). ESBLs are mainly associated with the family Enterobacteriaceae, of which *E. coli* and *Klebsiella pneumoniae* are the main ESBL producers [43]. This was obvious in our results where *E. coli* was by far the most predominant bacteria in all specimen types, followed by *Klebsiella* spp. and to a lesser extent *Enterobacter* spp. (Table 3). A study on hospitalized patients in Jordan found that from 121 *E. coli* isolated from urinary tract, 62% were ESBL producers [44]. Another study reviewed 1718 published studies worldwide, and reported the prevalence of ESBL-producing bacteria in pediatric blood stream infections to range from 4% in Europe to 15% in Africa [4]. This study observed an annual increase in the prevalence of ESBL-producing bacteria over the period 1996 to 2013 to be 3.2% [4]. In the past, infections complicated by ESBL were restricted to hospitals, nowadays they have spread to the community [43]. ESBL-producer dissemination is not only limited to clinical cases, it has also been detected in hospital environments,

household settings and even in vegetables and meat [45,46].

4. Conclusions

Clinical laboratory data is a vital tool in identifying the causative agents of infections and their associated resistance to antimicrobials. Retrospective review of clinical microbiology laboratory data can be a rich source of information to study the dissemination of microorganisms in specific population groups or certain body systems. This can aid epidemiologists in identifying trends in the isolation of specific microorganisms and it helps clinicians in revising their treatment protocols and guidelines to a more appropriate ones.

Acknowledgement

The authors would like to thank the workers in the Microbiology laboratories and the computer centers of the two hospitals for their help in collecting the required information..

Table 1: Details of the specimens tested and their distribution within gender, age, source and type.

Factor	Frequency (%)
Gender (N=5547)	
Male	1393 (25.1)
Female	4154 (74.9)
Age (Years; N=5547))	
≤1	250 (4.5)
1<Age<5	150 (2.7)
5-12	400 (7.2)
13-17	169 (3.0)
18-50	2515 (45.3)
51-70	1184 (21.3)
≥71	879 (15.8)
Specimen source (N=5547)	
Blood	98 (1.8)
High vaginal swab	81 (1.5)
Respiratory system	143 (2.6)
Skin, wound, abscess	1123 (20.2)
Urine	3924 (70.7)
Other sources including unidentified sample source	178 (3.2)
Isolates (N=7143)	
Gram-positive bacteria N=3714 (52.0%)	
Enterococcus	1342 (18.8%)
Staphylococcus	1249 (17.5%)
Streptococcus	1123 (15.7%) (*Strep* GpB n=1069)
Gram-negative bacteria N=3236 (45.3%)	9 (0.1%)
Acinetobacter	5 (0.07%)
Citrobacter	146 (2.0%)
Coliform	20 (0.3%)
Enterobacter	2290 (32.1%)

Escherichia coli	611 (8.6%)
Klebsiella	61 (0.9%)
Proteus	74 (1.0%)
Pseudomonas	20 (0.3%)
Others	154 (2.2%)
Yeast (*Candida*)	39 (0.5%)
Fungus	
ESBL-producing Bacteria N=1843	**Number (%) of ESBL producers**
	11 (0.6%)
Enterobacter	1481 (80.4%)
Escherichia coli	338 (18.3%)
Klebsiella	8 (0.4%)
Proteus	

Table 2: The distribution of the microorganisms amongst the specimens and gender and the association of the microorganisms with gender, if present, p< 0.05. The number of isolates of each microorganism obtained from females (F) or males (M) is shown.

Microorganism	Urine (N=3924) n (%)	SWA N=1123 n (%)	Respiratory (N=143) n (%)	Blood (N=98) n (%)	High vaginal swab (N=81) n (%)
***Enterococcus* spp. (N=1342)**	1334 (34%)	5 (0.02%)	0	1	0
Gender distribution	F= 1133, M=201 p<0.005 (F)*	F= 2, M= 3 -	-	F=0, M=1 -	-
Streptococci (N=1123)	194 (4.9%)	784 (69.8%)	68 (47.6%)	15 (15.3%)	37 (45%) all GBS
Gender distribution	F=165, M=29, p=0.043 (F)*	F=486, M=198, p<0.005 (M)*	F=29, M=39 p= 0.517	F=6, M=9 p=0.409	-
Staphylococci (N=1249)	1186 (30.2%)	37(3.3%)	7 (4.9%),	10 (10.2%),	0
Gender distribution	F=997, M=189 p<0.005 (F)*	F=24, M=13 p=0.701	F=3, M=4 p=1.00	F=3, M=7 p=0.195	-
***Escherichia coli* (N=2290)**	2028 (51.7%)	146(13.0%)	17 (11.9%)	34(34.7%)	16 (19.8%)
Gender distribution	F=1570, M=458 p<0.005 (M)*	F=125, M=21 p<0.005 (F)*	F=3, M=14 p=0.064	F=17, M=17 p=1.00	-
***Klebsiella* spp. (N=611)**	464 (11.8%)	60 (5.3%),	14 (9.8%),	30 (30.6%)	5 (6.2%)
Gender distribution	F=348, M=116 p=0.015 (M)*	F=51, M=9, p<0.005 (F)*	F=8, M=6 p=0.25	P=0.05 -	-

Microorganism	Urine (N=3924) n (%)	SWA N=1123 n (%)	Respiratory (N=143) n (%)	Blood (N=98) n (%)	High vaginal swab (N=81) n (%)
***Pseudomonas* spp. (N=74)**	45 (1.1%)	17 (1.5%)	8 (5.6%)	0	0
Gender distribution	F= 34, M= 11, p=0.532	F= 13, M=4 p=0.603	F= 4, M= 4 p=0.71		-
***Proteus* spp. (N=61)**	50 (1.3%)	6 (0.5%)	2 (1.4%)	0	1 (1.2%)
Gender distribution	F= 41, M=9 p=0.728	F= 6, M= 0 P= 0.185	F= 1, M= 1 -		-
***Candida* spp. (N=154)**	36 (0.9%)	59 (5.2%)	24 (16.8%)	1 (1%)	26 (32.1%)
Gender distribution	F= 29, M=7 p= 1.00	F= 49, M= 10, p=0.01 (F)*	F= 11, M= 13, p=0.648	F= 0, M=1 -	-
***Acinetobacter* spp. (N=9)**	0	4 (0.4%)	3 (2%)	0	0
Gender distribution	-	F=1, M=3 -	F=0, M=3 -	-	-
***Citrobacter* spp. (N=5)**	2 (0.05%)	2 (0.2%)	0	0	0
Gender distribution	F=2, M=0 -	F=2, M=0 -	-	-	-
***Enterobacter* spp. (N=20)**	7 (0.2%)	7 (0.6%)	1 (0.7%)	0	0
Gender distribution	F=4, M=3 p=0.16	F=5, M=2 p=1.00	F=0, M=1 -	-	-

* Statistically significant association was found with females (F) or males (M)

Table 3: The distribution of ESBL producing bacteria in the specimens collected based on the specimen source.

Microorganism	Urine (N=3924)	Blood (N=98)	High vaginal swab (N=81)	Respiratory (N=143)	SWA (N=1123)
***E. coli* (N=1481)**	1350 (34.4%)	33 (33.7%)	7 (8.6%)	16 (11.2%)	28 (2.5%)
***Klebsiella* spp. (N=338)**	236 (6.0%)	28 (28.6%)	2 (2.5%)	9 (6.3%)	25 (2.2%)
***Enterobacter* spp. (N=11)**	6 (0.2%)	0	0	0	1 (0.1%)
***Proteus* spp. (N=8)**	6 (0.2%)	0	0	0	1 (0.1%)

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عزلات المختبرات السريرية الجرثومية: إختلاف هيمنتها وإرتباطها بجنس المريض

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ملخص

يعتبر أرشيف البيانات للمختبرات السريرية الجرثومية مصدر مهم وقيم للمعلومات. في هذه الدراسة، تم مراجعة بيانات هذه المختبرات من مستشفيين متخصصين في الأردن من أجل تحديد مصدر العينات للجراثيم الأكثر انتشاراً ولمدى تفشي بكتيريا المنتجة للبيتالاكتيميز الواسع الطيف. كذلك، تم تقصي وجود علاقة بين جنس المريض والجراثيم المعزولة منه أو مصدر هذه الجراثيم. بعد الإطلاع على البيانات من المستشفيات وفرزها بناء على مواصفات محددة ، تم تحديد 5547 عينة فيها نمو جرثومي مؤكد من أصل 20726 ملف تمت دراستهم. بهذه العينات، وجد 7143 عزلة تم التعرف عليها لمستوى الجنس والنوع. تم تحديد أكثر العينات شيوعاً مع إجراء دراسة إحصائية لوجود إرتباط لها بجنس المريض، ومناقشة النتائج .تم مقارنة هيمنة جراثيم معينة بعينات معينة ومحاولة تفسير هذه النتائج وتوضيحها من خلال المعلومات والأبحاث المنشورة. بالإضافة لذلك، وجدت بعض العلاقات بين جنس المريض ونوع الجراثيم المهيمنة بمصادر معينة للعينات. وجد انتشار عالي للبكتيريا المنتجة للبيتالاكتيميز الواسع الطيف (57%) ضمن البكتيريا سالبة الجرام، ما يدل على خطورة التحديات التي تواجه الأطباء بإيجاد علاجات مناسبة. إن مراجعة بيانات المختبرات السريرية الجرثومية يساعد في دراسة التوجه في إنتشار الجراثيم ضمن فئات بشرية معينة وكذلك يساعد الأطباء في تعديل البروتوكولات العلاجية.

الكلمات الدالة: إنتيروكوكس، ستربتوكوكس أجالاكتي، بكتيريا المنتجة للبيتالاكتيميز الواسع الطيف، جنس، هيمنة، مختبر، زراعة البول، مسحة المهبل.

تاريخ استلام البحث 2020/7/12 وتاريخ قبوله للنشر 2021/4/30.

Hospital Staff Perspectives toward Medication Reconciliation: Knowledge, Attitude and Practices at A Tertiary Teaching Hospital in Jordan

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ABSTRACT

Medicine reconciliation (MedRec) is a patient safety goal. A cross-sectional study using a self-completed questionnaire was conducted at the Jordan University Hospital (JUH) in October- December 2016. A convenient sample of physicians, pharmacists, and nurses was targeted. Statistical analysis was performed to outline the variances across professional groups and factors associated with knowledge, attitude, and practice. Two hundred questionnaires were analyzed of which 41 (20.5%) were completed by physicians, 23 (11.5%) by pharmacists, and 136 (68.0%) by nurses. In total, 162 (81.0%) of the participants responded that they had heard about MedRec before the questionnaire, and139 (69.5%) were aware of the existing policy for MedRec and 143 (71.5%) knew the requirements to complete the MedRec form. Nurses were more likely to know about the MedRec policy and the requirements to complete the MedRec form ($p = 0.034$ and 0.041 Chi Square, respectively). For 119 (73.5%), working at the JUH was the main source to know about MedRec participants. Level of education appeared significantly to influence knowledge about MedRec. Those holding a postgraduate degree were more likely to know about MedRec 123 (61.5%), $p = 0.01$, Chi Square test). Moreover, increased years of practice at the JUH significantly influenced knowledge about MedRec ($p = 0.04$, Mann Whitney test). The MedRec attitude scores median (IQR) was 10 (9-12) indicating supportive views across the study profession groups. None of the variables significantly influenced attitudes toward MedRec. The median (IQR) of MedRec practice was 10 (8-13) representing moderate (62.5%) practice score. MedRec practice scores were significantly affected by profession type ($p = 0.00$, Kruskal Wallis test). Most often117 (66.8%), the MedRec was completed only upon admission and information about therapy changes were less often recorded compared to patient and medical history details. One third of participants reported they often relied on one source of information, mainly family member or caregiver 117 (66.8%). In conclusion, physicians, nurses and pharmacists in an accredited teaching hospital in Jordan were found to knew about MedRec, policy and form completion requirement. All profession groups showed supported views toward MedRec. However, the practice of MedRec was moderate and physicians followed by nurses were more involved in MedRec steps compared to pharmacists. MedRec often performed upon admission and focus on patient history taking. Practice of MedRec should be enhanced to achieve patient safety centered goals rather than accreditation centered purposes.

Keywords: Medicine reconciliation; Jordan; KAP; health care continuity, patient safety, hospital accreditation.

INTRODUCTION

Patient transfers across health settings presents increased opportunity for transition errors. On average 60% of medication errors occur during patient admission, discharge, or transfer of care. Additionally, over 50% of patients experience at least one discrepancy at the point of transfer and some are found to continue post-discharge.[1]

Received on 2/12/2020 and Accepted for Publication on 21/5/2021.

Medication reconciliation (MedRec) is recognized as a patient safety goal by the Joint Commission for Health Organization Accreditation (JC) since 2006.[2] Consequently, accredited health organizations are urged to demonstrate the practice of MedRec for an accreditation award and renewal. MedRec consists of a set of steps, namely, collecting and creating the most possible accurate list of all medications a patient is taking, and then communicating that information to the next health care provider.[2] Previous international studies outlined that MedRec might not fully recognized or practiced due ambiguity in the MedRec process, responsibility and applicability across organizations [3, 4, 5 6, 7, 8, 9, 10, 11] Jordan is no exception. At four tertiary hospitals accredited by JC, pharmacists' perceptions toward MedRec practice were investigated and were found to be suboptimal. Less than 50% of pharmacists were aware of the presence of MedRec policies in the hospital and MedRec was implemented for most admitted patients.[12] A recent study in Kuwait, assessed the perceptions of physicians and pharmacists at six governmental hospitals and reported that physicians and pharmacists were aware of MedRec terms, but the practice of MedRec was found again suboptimal.[13] Perceptions of the nursing team were not captured in existing studies which underlines needs for further investigation. This study aimed to investigate knowledge, attitude, and practices (KAP) of physicians, pharmacists, and nurses toward MedRec at an internationally accredited renowned teaching hospital in Jordan.

Method:

Study Design and Questionnaire

A cross-sectional study using a self-completed questionnaire was conducted at the Jordan University Hospital (JUH). The study commenced on October 2016 and lasted for two months. Three hundred and forty questionnaires were distributed to a convenient sample of physicians, pharmacists, and nurses. They were approached at various days, times, and locations. The JUH was accredited by the JC in 2007 and has adapted a MedRec form that is attached to all admitted patient files.

The study questionnaire which was developed after an extensive literature review [7, 8, 10, 12, 13, 14] was face validated and piloted before wide distribution. The questionnaire consisted of four parts assessing the knowledge, attitudes, and practices of MedRec. Demographic data were also collected. The questionnaire is available upon request.

Responses to the questions were Yes/No answers, multiple-choice, and Likert scale depending on the nature and scope of the question.

Statistical Analysis

The data were coded, entered, and analyzed using the Statistical Package for Social Sciences (SPSS, Version 22). The descriptive analysis was summarized as mean (SD) and median and interquartile range (IQR) as appropriate. Frequencies and percentages were used for categorical variables. A non-parametric test was used where data were not normally distributed. Normal distribution was checked visually and using Shapiro-Wilk test, with p-value ≥ 0.05 indicating normally distributed continuous variables.

The MedRec attitude score was calculated scoring six statements. With attitude scores ranging from 0-12, 2 for agree, 1 uncertain and 0 for disagree. Higher scores represented better attitudes. A score of 80% or higher was considered to represent high supportive attitudes whilst a score of 60 to 79% represented moderate attitudes, and a score below 60% represented low supportive views. Similarly, a score representing MedRec practices was calculated by scoring each response as either "agree" two points, "uncertain" one point, and "disagree" zero points. Thus, the practice scores ranged from 0-16 with 16 representing a 100% practice score. A score of 80% or higher was considered to represent a high satisfactory practice, whilst a score of 60 to 79% represented moderate practice, and a score below 60% was considered to represent unsatisfactory practice.

Univariate analysis contributing to knowledge, attitude, and practice was performed. Chi Square, Mann-

Whitney test, Kruskal Wallis tests, and Fischer exact tests were used as appropriate. Bivariate Spearman correlation was used to assess the factors influencing practice and attitude scores. A P-value of less than 0.05 was considered statistically significant.

Ethical Approval

Scientific and ethical approval was obtained from the Research and Scientific Committee at the School of Pharmacy at the University of Jordan and the Institutional Review Board committee at the JUH, number 198/2016 on 27th Sep 2016.

RESULTS

Study Sample

A total of 210 questionnaires were returned with a response rate of 61.8%. Ten questionnaires were excluded due to missing data about the profession of the participant who completed the questionnaires. Therefore, 200 questionnaires were included in the analysis, of which 41 (20.5%) were completed by physicians, 23 (11.5%) by pharmacists, and 136 (68.0%) by nurses. The majority of the study participants were females for 109 (55.0%). The median (IQR) age was 27 (25-30) and 159 (79.5%) had a first university degree (BSc/MD). They mostly graduated from public universities for 153 (76.5%). The median (IQR) of practice at the JUH was 4 (2-6) years. Table 1 summarizes the study sample characteristics per profession type.

Table 1: Characteristics of the study sample (N=200) and knowledge of MedRec, policy and hospital form

Characteristics	Physicians (N=41)	Pharmacists (N=23)	Nurses (N=136)
Gender			
Female	18 (43.9%)	14 (60.9%)	77 (56.6%)
Age years			
Median (IQR)	27 (25-28)	29 (24-36)	28 (26-31)
Education Level			
Diploma	0 (0.0%)	2 (8.7%)	9 (9.6%)
BSc/MD	30 (73.2%)	19 (82.6%)	110 (80.9%)
Master	11 (26.8%)	2 (8.7%)	17 (12.5%)
University/ College of graduation			
Public	38 (92.7%)	14 (60.9 %)	101 (74.3%)
Years of practice at JUH			
Median (IQR)	3 (1-4)	5 (1-8)	4 (2-8)

IQR, interquartile range

Knowledge about MedRec

Table 2 present knowledge about MedRec, existing policy and requirement to complete the MedRec form as well as source of information about MedRec per profession type. In total, 162 (81.0%) of the participants had heard about MedRec before the questionnaire, whilst 139 (69.5%) responded that they were aware of an existing policy of MedRec at the JUH and 143 (71.5%) knew the requirements to complete the MedRec form for every patient. Nurses were more likely to know about the MedRec policy and the requirements to complete the MedRec form ($p = 0.034$ and 0.041 Chi Square, respectively). Working at the JUH was the main source of knowledge about MedRec for 119 (73.5%) participants, followed by hospital training for 39 (24.1%) and during their study for 19 (11.7%).

Table 2: Knowledge about MedRec, existing policy and requirement to complete the MedRec form as well as source of information about MedRec per profession type

Knew about	Physicians (N=41)	Pharmacists (N=23)	Nurses (N=136)	P value
MedRec	30 (73.2%)	19 (82.6%)	113 (83.1%)	0.04
MedRec policy at JUH	24 (58.5%)	13 (56.5%)	102 (75.0%)	0.02
Requirement to complete MedRec form existence	25 (61.0%)	12 (52.2%)	106 (77.9%)	0.03
Source of information about MedRec				
During my study	3 (10.0%)	5 (26.3%)	24 (20.7%)	0.32
During my training	7 (23.3%)	10 (52.6%)	22 (19.5%)	0.07
A word from a colleague	1 (3.3%)	2 (10.5%)	3 (2.7%)	0.10
During work at JUH	21 (70.0%)	8 (42.1%)	90 (79.6%)	<0.001

Factors contributing to Knowledge about MedRec, policy and requirements for the MedRec form

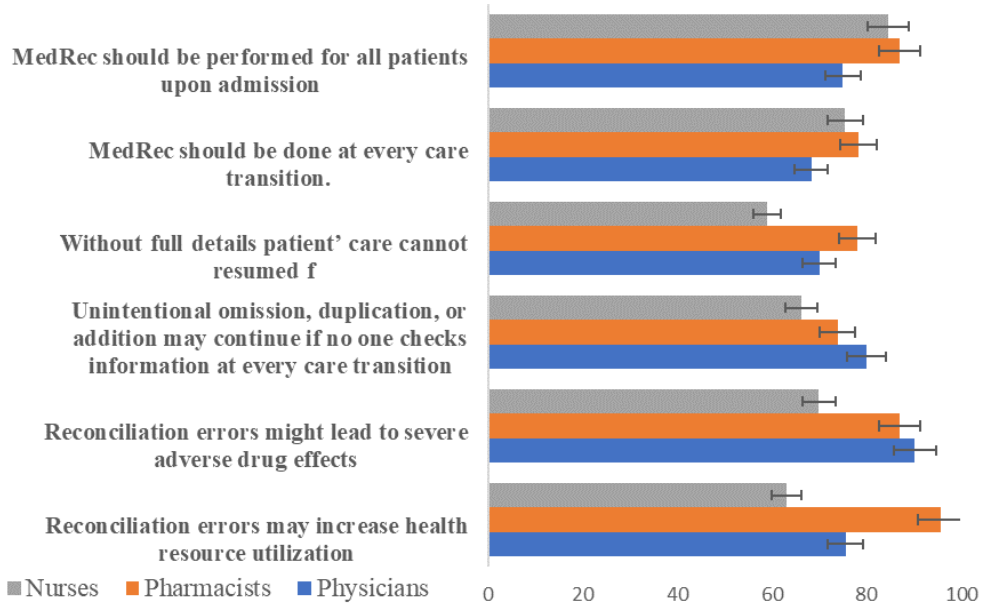
Education level demonstrated significant association with the knowledge about MedRec ($p = 0.01$, Chi Square test). With those holding a postgraduate degree across profession groups being more likely to know about MedRec for 123 (61.5%, $p = 0.01$, Chi Square test). Moreover, years of practice at the JUH significantly influenced knowledge about MedRec ($p = 0.04$, Mann Whitney test); the median (IQR) was 4 (2-8) practice years at the JUH for those who had heard about MedRec during work at the JUH. There was also a significant association between profession type and whether participants knew about MedRec through work at the JUH ($p < 0.001$, Chi Square test) as it can be seen Table 2.

There was only a significant association between profession type and the knowledge of the MedRec policy and requirements to complete a designated form. Nurses were more likely to know about the policy. As for the requirement to complete MedRec form upon patient admission, nurses were also more likely to know about such requirement. A significant association was found too between the knowledge of the policy and years of practice at the JUH ($p = 0.00$, Mann Whitney test). The median (IQR) was 4 (2-8) practice years at the JUH for those who knew about MedRec policy compared to 2 (1-3). Similarly, nurse participants and those with more years of practice at the JUH were more likely to know about the requirements to fill the MedRec form for each admitted patient (p value < 0.01)

Attitude toward MedRec

The median (IQR) of attitude score was 10 (9-12) indicating supportive views across profession groups. Figure 1 presents agreement with the MedRec attitude statements per profession type. The median (IQR) of attitude score for physicians was 10 (9-12), for pharmacists 9 (9-11) and for nurses 10 (10-12), $p=0.42$.

None of the variables significantly affected attitudes toward MedRec.



Practice of MedRec

Table 3 presents MedRec steps performed per profession type. The median (IQR) of MedRec practice was 10 (8-13) representing 62.5% of the practice score. MedRec practice scores were significantly associated with profession type (p = 0.00, Kruskal Wallis test); The MedRec practice score median (IQR) was 12 (8-14) for physicians, 8 (6-9) for pharmacists, and 11 (8-13) for nurses.

Overall, 65 (32.5%) of the participants reported that they use only one source to verify medication history, 41 (30.1%) use two sources, and the remainder reported using three sources or more. A family member or caregiver was the main source of information for 117 (66.8%), followed by medications brought with patients from home for 108 (61.7%), previous discharge summary for 101 (57.7%), and previous prescription for 71 (40.6%).

As per profession type, 22 (53.7%), 7 (30.4%), and 55 (40.4%) of the physicians, pharmacists, and nurses, respectively, recalled identifying reconciliation errors following a care transition in the past six months.

Table 3: MedRec Practice at JUH per profession type

MedRec step	Physicians (N=41)	Pharmacists (N=23)	Nurses (N=136)	P*
Interviewing all patients upon admission to obtain medication history.	34 (82.9%)	13 (56.5%)	112 (82.4%)	0.03
Verifying medication histories from multiple sources	40 (97.6%)	14 (60.9%)	121 (89.0%)	0.05
Verifying medication history with inpatient medication list	28 (68.3%)	14 (60.9%)	109 (80.1%)	0.05
Discussing identified discrepancies (omission, duplication, addition) with care team	27 (65.9%)	15 (65.2%)	61 (44.9%)	0.10
Providing name and phone to be contacted in case of any question	5 (12.2%)	3 (13.0%)	22	0.63

when patient transfers from your care			(16.2%)	
Providing a written summary on patient's admission and medication information to share with next health care provider	33 (80.5%)	5 (21.7%)	81 (59.6%)	0.01
Verifying discharge medications with inpatient medications upon discharge to ensure all changes to medications are documented	25 (61.0%)	10 (43.5%)	70 (51.5%)	0.07
Verifying discharge medications with preadmission medication history to ensure no omissions or duplications or discontinuation	16 (39.0%)	6 (26.1%)	65 (47.8%)	0.34

*Chi squared test

Table 4 presents the time when each profession completed a MedRec form. Often, MedRec form was completed upon admission.

Table 4: Completion of MedRec form

Time of completion	Physicians (N=25)	Pharmacists (N= 12)	Nurses (N=106)	Total (N=143)
Upon admission	21 (84.0%)	6 (50.0%)	96 (90.6%)	123 (86.0%)
Upon discharge	4 (16.0%)	1 (8.3%)	11 (10.4%)	16 (11.2%)
Upon transfer between wards	1 (4.0%)	2 (15.4%)	16 (15.1%)	19 (13.3%)
Every time a medication changed	3 (12.0%)	1 (8.3%)	14 (13.2%)	18 (12.6%)

*As participants were allowed to choose more than one choice, total percentages may exceed 100%

Moreover, 84 (42.0%) of the participants encountered reconciliation errors in the last 6 months following a care transition. Of them, 25 (29.8%) believed that the identified reconciliation errors were most likely unintentional, whilst 41 (48.8%) believed they were sometimes unintentional and only 18 (21.4%) believed they were rarely unintentional.

Information filled in a MedRec form per profession is presented in Table 5.

Table 5: Information filled in MedRec form per profession type

Information in MedRec form	Physicians (N=25)	Pharmacists (N=12)	Nurses (N=106)
Patient details*	20 (80.0%)	6 (50.0%)	85 (80.2%)
All medications taken at home	15 (60.0%)	7 (58.3%)	72 (67.9%)
All medications taken in the hospital	16 (64.0%)	8 (66.7%)	56 (52.8%)
All medications to take home after discharge	11 (40.0%)	3 (25.0%)	44 (41.5%)
Medication generic name	14 (56.0%)	6 (50.0%)	59 (55.7%)
Medication trade name	13 (52.0%)	5 (41.7%)	64 (60.4%)

Dose	17 (68.0%)	7 (58.3%)	78 (73.6%)
Route	15 (60.0%)	5 (41.7%)	85 (80.2%)
Frequency	17 (68.0%)	7 (58.3%)	79 (74.5%)
Start date	9 (36.0%)	2 (16.7%)	66 (62.3%)
Discontinuation date (if applicable)	8 (32.0%)	3 (25.0%)	47 (44.3%)
Known allergies	15 (60.0%)	6 (50.0%)	84 (79.2%)
Intended length of courses e.g. antibiotics	9 (36.0%)	3 (25.0%)	46 (43.4%)

* Name, date of birth, file number, etc.

Factories contributing to MedRec practice

MedRec practice scores were significantly affected by gender (p = 0.01, Mann Whitney test); the MedRec practice scores median (IQR) was 11 (8-14) for females compared to 10 (7-12) for males. Moreover, the educational level significantly influenced MedRec practice scores (p = 0.01, Kruskal Wallis test); participants with a master degree had MedRec practice scores median (IQR) 11.5 (8-14) compared to 10 (8-13) with a bachelor degree and 6 (4-12) with a diploma degree. Furthermore, although the p values of the spearman correlation analysis between MedRec practice scores and age or MedRec practice scores and JUH practice years were both 0.02; the spearman's coefficient between MedRec practice scores and age or MedRec practice scores and JUH practice years were -0.18 and -0.17, respectively. This indicates that the correlations between MedRec practice scores and age or MedRec practice scores and JUH practice years are negligible.

Discussion

This study evaluated the knowledge, attitude, and practice of the healthcare staff toward MedRec in an internationally accredited teaching hospital. The majority of health staff knew about MedRec, policy, and requirements for form. The year of practice in the study site and post-graduate training were contributed significantly better knowledge of MedRec, policy and the hospital form. Of note, pharmacists reported their study as the main source to know about MedRec, whilst physicians and nurses reported work as the leading source of information. Additionally, nurses were more likely to know about the MedRec policy and form completion requirements.[15] This might reflect high involvement of nurses in the accreditation office. Future evaluation whether involvement in administrative and accreditation roles might catalyst knowledge and practice of MedRec is recommended.

In line with previous reports, [8,10,14-16] there was a consensus among participants that MedRec implementation is significant to patient care and safety and should be performed at each care transition. [6, 8, 12, 13, 14] The majority of participants agreed that the discrepancies of unintentional omission, duplication, or addition to patient medications might continue if no one checks patients' information at every care transition. This outlines importance of MedRec effective implementation to ensure patient safety goals.[15, 16] Additionally, it is vital to promote continues training and education that promote the process and implantation of MedRec during undergraduate and postgraduate levels.[15]

Although MedRec as a process is well defined, the practice and optimum implementation of MedRec appears to represent a major challenge for health institutions worldwide.[4, 8, 17, 18, 19] We found MedRec practice moderate in one of the leading JC accredited teaching hospitals. This was outlined previously by other institutions.[6, 7, 12, 15] These studies outlined that full MedRec was not implemented to most patients and the best possible medication history was not constructed at patient admission. Additionally, patient and therapy information were not clearly communicated or documented.[6, 7, 12, 17, 18] Reasons for suboptimal implementation of MedRec and strategies for effective

implementation might vary across institutions.[6, 13, 15] Therefore, standardization of MedRec practice across organizations and health settings might not be possible.[13, 19] Nonetheless, it is important to underline institutional and human barriers and facilitators through continual meetings and discussions between managers and practitioners on an organization level as well as local and international levels.[4]

MedRec practice varied across profession groups with physicians and nurses reporting more involvement in MedRec steps in the study site compared to pharmacists. This is in contrast with the accumulating evidence underpinning most effective MedRec interventions as those involving pharmacists and highlighting leading roles of pharmacists with MedRec steps.[15, 18, 19]Factors hindering pharmacist involvement in MedRec are worth investigation in Jordan. [12, 13, 18, 20] Of note, 11 of respondents to the study questionnaire we pharmacy and nursing assistants (Diploma holders), these were seniors and involved in similar extents of patient care to BSc staff. This highlights variances in the staff performing MedRec across organization and outline needs to understand better facilitators and barriers for each profession.

Deficiencies in information completed in the MedRec form and the less focus on therapy changes information needs careful attention. Lack of such practice might present a risk for unintentional discrepancies to be continued resulting in potential patient harm and treatment costs.[4, 8, 11, 17] In addition, the MedRec forms mostly completed upon admission and those information left un-reconciled might be carried until discharge. Effective and optimum implementation on the organization level requires meetings and discussions between professions, practitioners, and managers to ensure continuous evaluation of the process. Thus, it is recommended for accreditation bodies to focus on mapping the MedRec process across organizations to ensure applicability and feasibility.[21] Onsite evaluation for accreditation renewal should be linked to organization compliance with active implementation of MedRec. This requires defining MedRec practice indicators and assessing the quality of information sharing at every point of patient transition.

Limitations

This is a single sited study using convenient sampling techniques. Thus, there is an inherent selection bias. The generalizability to other settings or unaccredited facilities can be enhanced by multi-center study design and further statistical validation of the study tool to ensure adaptability across organization. However, this study scoped the perspectives of physicians, pharmacy, and nursing staff and outlined factors associated with knowledge, attitude, and practice of MedRec. Future studies to investigate causative links via regression analysis between MedRec perception and practice across health organizations are recommended.

Conclusion

Members of the medical team in an accredited teaching hospital in Jordan knew about MedRec, its policy, and its form. All professional groups reported supportive attitudes toward MedRec. MedRec practice was found moderate and mainly in the form of history taking. Therapy changes and contact information were often not reconciled fully. Reasons underlying the perceptions and practice across profession groups should be investigated. Health accreditation bodies and mangers should map MedRec process often to ensure effective implementation .

Acknowledgements

The authors would like to thank the participants who completed the questionnaire. Moreover, the authors would like to thank the Deanship of Academic Research at the University of Jordan for funding this project.

Conflict of interest

All authors wish to declare no conflict of interest.

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وجهات نظر الطاقم الطبي بما يتعلق ب التوافق الدوائي: المعرفة و التطبيق في مستشفى تعليمي متخصص في الاردن

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ملخص

التوافق الدوائي هو متطلب لأمن و سلامة المريض و استمرارية العناية بالمريض. تم اخذ عينة مقطعية و توزيع استبيان في مستشفى الجامعة الاردنية لأطباء و صيادلة و ممرضين في مواقع متعددة و اوقات دوام وتخصصات من المستشفى. تم تحليل الاستبيانات تحليلا وصفيا و سببيا لتحديد التنوع بالآراء و المواقف بين التخصصات. مئتان استبيان تم تعبئتهم بشكل كامل وتم تحليلهم. معظم المشاركين من مختلف التخصصات يعرفون عن سياسات و نماذج تطبيق التوافق الدوائي في المستشفى. الممرضين كانوا الاكثر معرفة من حيث السياسات و الخطوات. أجمعت التخصصات ان تطبيق التوافق الدوائي في غاية الاهمية لتحقيق رعاية و سلامة المرضى و يجب تطبيقه بكل حالات انتقال المريض. ممارسة التوافق الدوائي كانت متوسطة و كان الاطباء و الممرضين اكثر مشاركة في التوافق الدوائي مقارنة مع الصيادلة. المعلومات المتعلقة بتغيرات الدواء في معظم الاحيان لا تعبأ في النموذج المخصص. اعضاء الفريق الطبي من اطباء و صيادلة و ممرضون يعرفون عن التوافق الدوائي و سياساته في المستشفى . و لكن تطبيق التوافق الدوائي غير كامل . جميع التخصصات اجمعت على اراء ايجابية بما يتعلق بأهمية التوافق الدوائي. البحث عن الأسباب مهم لتحسين الاداء و التطبيق في المستقبل.

الكلمات الدالة: التوافق الدوائي، الأردن.، المعرفة و الممارسة و الآراء، تواصل الرعاية الصحية.

تاريخ استلام البحث 2020/12/2 وتاريخ قبوله للنشر 2021/5/21.

Jordan Journal of Pharmaceutical Sciences, Volume 14, No. 2, 2021

The effects of pomegranate juice on monocrotaline-induced hypertensive pulmonary vascular changes and right ventricular hypertrophy in rats

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ABSTRACT

Pulmonary hypertension is associated with structural pulmonary vascular changes. Due to the increase in pulmonary vascular resistance, right ventricular hypertrophy and right heart failure develop. Previous studies have shown that pomegranate juice has anti-inflammatory and anti-proliferative effects. The purpose of this study was to determine whether pomegranate juice intake could prevent or reverse changes in pulmonary circulation in a monocrotaline-induced pulmonary hypertension rat model. Pulmonary hypertension module was established in Westar rats by a single subcutaneous injection of monocrotaline (60mg/kg). Experimental groups were as follows: control, monocrotaline for 3 weeks, monocrotaline +Pomegranate juice for 3 weeks, and monocrotaline for 3 weeks followed by Pomegranate juice for 3 weeks (n=10 per group). The pathohistological changes of pulmonary arteries, right ventricular hypertrophy caused by monocrotaline, and pomegranate juice were analyzed. The thickness of the pulmonary arterioles and right ventricular are reduced significantly by the intake of pomegranate juice. Giving it with monocrotaline at day zero resulted in less thickening of pulmonary arterioles, alveolar septa, and right ventricle wall. We conclude that pomegranate juice intake ameliorates the pulmonary vascular remodeling developed in monocrotaline-induced pulmonary hypertension.

Keywords: Monocrotaline; Pomegranate juice; Right ventricular hypertrophy; Pulmonary blood vessels.

INTRODUCTION

Pulmonary hypertension (PH) is associated with a variety of adult and pediatric diseases, however, it is characterized by common features, such as sustained pulmonary vasoconstriction due to increased vascular tone and progressive structural remodeling of pulmonary arteries (1). Patients with severe PH have combinations of small pulmonary arteries adventitial and medial thickening, occlusive intimal lesions, and obliterating thrombotic and plexiform lesions (2). Pulmonary hypertension follows a progressive and fatal course due to the development of right ventricular (RV) dysfunction and failure (3). Several models have been developed and extensively studied over the years, yet the most commonly used animal models are rodents exposed to either hypoxia or monocrotaline (4). Monocrotaline (MCT), an organic alkaloid extract of the plant Crotalaria Spectabilis, induces mild-moderate increases in pulmonary arterial pressure (30-40 mmHg), most prominently 3-4 weeks after injection (5). Pulmonary vascular remodeling is more severe than that found in chronic hypoxia-exposed rats, however the more complex occlusive concentric laminar and plexiform lesions are lacking (6).

The high mortality in PH patients necessitates an in-depth evaluation of the available treatments. In 2007,

Received on 8/3/2021 and Accepted for Publication on 21/5/2021.

Macchia and colleagues performed an extensive meta-analysis of clinical trials on PH, which revealed a statistically insignificant 30% reduction in mortality in patients receiving experimental treatments (7). The same group showed in 2010 that the "pooled" effect of all treatments had a significant 39% reduction in "all-cause" mortality, although no class of drugs, per se, produced a statistically significant reduction (8). These disappointing results reveal the inadequacy of the current treatments of PH, which, in turn, mandates more effort in finding new, more effective drugs and a wider range of therapeutic options.

Although there is general agreement on at least three components of PH pathogenesis: sustained pulmonary vasoconstriction, progressive structural remodeling and occlusion of small pulmonary arteries and arterioles, and in-situ thrombosis, the relative contribution, chronology, and triggers of these processes have not yet been elucidated (9). Patients with severe PH have combinations of small pulmonary artery medial wall thickening, and occlusive concentric neointimal and plexiform lesions (10). Such complex pathology probably reflects the multifactorial nature of PH, which means several signaling pathways are aberrant in the process. At present time and despite the development of many drugs, there is no single fully effective therapy (11). Given the history of poor patient compliance for using multiple drugs for the same problem, it is logical to start evaluating multifunctional compounds that can, in theory, achieve the same results as a combination of drugs.

Pomegranate fruit has been used as a medicinal substance by ancient civilizations such as Greeks and Egyptians (12). Pomegranate juice (POM) is rich in polyphenols like punicalagin which is the main phytochemical ingredient of POM, which has many pharmacological properties due to its high antioxidant punicalagin (13). The antioxidant activities of POM are three times more than those found in red wine and green tea (14). The hemodynamic indicators, like PH and right ventricular hypertrophy, in hypoxia-induced PH in rats were improved by treatment with punicalagin (15). Other polyphenols like tannins and flavonoids are also found in POM, both of which have high antioxidant and anti-inflammatory activities. The oxidative stress was attenuated in mice and humans by intake of POM (16). POM juice supplementation reduces the size of atherosclerotic lesion by 44% and decreases foam cells number in apolipoprotein E- deficient mice. Also, Pomegranate juice has anti-proliferative effects (17) which could be attributed to its free radical scavenging properties. The anti-inflammatory properties of POM are through its inhibitory effect on some enzymes with relevant pharmacological properties like cyclooxygenase 2 which is needed for the synthesis of prostaglandin and leukotrienes (18). The anti-proliferative (17) and vasodilatory effects (19) of POM are also documented. Such a multifactorial nature of pomegranate makes it an attractive therapeutic target for disease states characterized by heterogeneous and complex pathogenic mechanisms like PH.

Based on the aforementioned background, we propose to test the benefits of POM by virtue of the antioxidant, anti-inflammatory, and vasorelaxation activities of its components in improving or preventing some features of PH and RV dysfunction/failure that are induced experimentally in a rat model of pulmonary hypertension and right ventricular hypertrophy induced by MCT. The results of this work will hopefully broaden our understanding of the PH through studying the effects of pomegranate extract on the animal model of PH. Moreover, confirmation of our working hypothesis may provide a novel mechanistic foundation for an effective, economical, and safe oral therapeutic approach for PH.

Materials and Methods:

Experimental procedures

All animal experimental protocols were reviewed and approved by the Jordan University of Science and Technology animal care and use committee (ACUC,

approval no. 2016/0315). Animals were treated in accordance with guidelines from the Care and Use of Laboratory Animals (8th edition, National Academies Press) and all procedures were confirmed to adhere to the mentioned guidelines by the ACUC. The experiments were performed on 40 male Sprague–Dawley rats (200-250 g) obtained from the university animal house. The rats were randomly divided into four groups (no=10 in each group) (Fig 1): the control group received a subcutaneous (SC) injection of normal saline, the Monocrotaline group (MCT) received a single SC injection of 60 mg/Kg to induce PH as published elsewhere (20), the prevention group (MCT + POM) received single SC injection of 60 mg/Kg on day one and pomegranate juice for 3 weeks, and finally the reversibility group (MCT/POM) received a single SC injection of 60 mg/Kg on day one and received pomegranate juice on day 21 to day 42 after injection of MCT. Since moncrotaline is metabolized in the liver and in higher doses more than 60 mg/kg could result in significant liver failure, we chose this does to induce pulmonary vascular disease with little non-pulmonary damage over 3 weeks.

Lung and Heart Tissue Preparation

Rat lungs and heart were resected via an open-chest procedure and washed in phosphate-buffered saline to remove blood. For each harvested heart, the RV free wall was quickly and carefully separated from the LV and intra-ventricular septum, and both parts were weighed. Fulton's index was calculated by dividing the RV weight by LV weight plus the septum weight (RV/LV+S) for the evaluation of RV hypertrophy. The right hilum was ligated, and the left lung was fixed for histology by tracheal instillation of a mixture of 1% formalin and 0.5% agarose under constant pressure (20 cm H_2O). The inflated lung was immersed in 10% formalin. Paraffin sections (5-µm thick) were obtained and stained with hematoxylin and eosin or anti-α-smooth muscle actin antibodies for immunohistochemical study.

Histomorphometric Analysis of Pulmonary Vascular Remodeling

Slides were analyzed using a light microscope by one of the authors in a blinded manner (OR). The microscopic images of intra-alveolar arteries with a diameter of 100-200 µm were analyzed. The luminal and total cross-sectional area of each examined vessel was obtained using a computerized morphometric program. From the previously mentioned values, the wall thickness was extrapolated. Wall thickness ratio (WT%) of pulmonary arterioles was determined from each animal (average of 15 vessels). WT% was calculated as the area occupied by the vessel wall divided by the total cross-sectional area of the arteriole. The tunica media (smooth muscle layer) of the blood vessel was visualized in anti-α-smooth muscle actin-stained slides, as it is an authentic marker for the medial layer.

Statistical Analyses

All values are expressed as mean ± standard deviation from the mean (SD). One-way analysis of variance (ANOVA) was used to determine the significance of differences between different groups and the significance of interactions between groups was determined by Tukey's post-hoc tests. P-values less than 0.05 were considered significant. Analyses were performed using Graph Pad Prism, version 8.

Results

Pulmonary Arterial Wall Thickness

Pulmonary arterial wall thickness % was significantly increased in the MCT group to 0.44 ± 0.03 % versus 0.22 ± 0.09 % in control group ($P < 0.05$) (**Fig.2**). With POM administration on the same day of MCT injection, the arterial wall thickness% had decreased to 0.35 ± 0.07 % ($P < 0.05$ vs MCT group). Giving POM 3 weeks after MCT injection did not prevent the increase in the thickness of the pulmonary arteriolar wall induced by MCT, the arterial wall thickness % was 0.42 ± 0.09 % which is not significantly different from the MCT group (**Fig.2**).

The increase in pulmonary arterial wall thickness (**Fig.3**) observed in the MCT group represents arterial tunica medial hypertrophy in these rats (**Fig.4**), which was prevented largely by the administration of POM at day zero.

Pulmonary alveolar septum thickness:

Pulmonary alveolar septum thickness was increased in the MCT group compared with the control group (**Fig.5**). With POM administration concurrently with MCT or after 3 weeks following administration of MCT, the alveolar septum thickness was decreased compared with the MCT group.

Ventricular hypertrophy assessment

RV Hypertrophy was estimated by changes in RV weight divided by LV+S weight ratio (Fulton index) (**Fig.6**) and examination of the ventricular wall thicknesses using H&E stained slides of the cross-sections of the heart taken 2 mm below the atrioventricular junction (**Fig.7**). The ratio was almost doubled by MCT treatment compared with the control group (0.22 ± 0.026 % versus 0.44 ± 0.09 % in the MCT group) and this was obvious in the cross-section of the heart. Administration of POM on the first day of MCT treatment succeeded in bringing down the ratio significantly (0.35 ± 0.07 % versus 0.44 ± 0.09 % in the MCT group). Administration of POM after three weeks of MCT administration failed to reduce the ratio significantly (0.42 ± 0.09 % in MCT and then POM group versus 0.44 ± 0.09 % in the MCT group). Body weight was significantly decreased in MCT group at 3 weeks compared to before administration of monocrotaline (190g ± 11.5 vs 231g ± 13.3, $P < 0.05$). Also, it was significantly reduced in MCT/POM group at six weeks compared to before administration of the moncrotaline (183g ± 11.5 vs 229g ± 13.3, $P < 0.05$).

Discussion:

Pulmonary hypertension is a fatal disease affecting mostly young people. It is associated with structural vascular changes in the pulmonary vessels. Concomitantly and due to the increase in the pulmonary vascular resistance, right ventricular hypertrophy and right heart failure develop. The burden of this disease is increasing worldwide with the lack of sufficient treatments and pharmacological options to be considered (7,8). Fortunately, animal models of pulmonary hypertension can be induced by different methods and the changes shown in these models are comparable to those changes seen in humans (4, 5, 6). In this study, we investigated whether the structural changes in pulmonary arteries and arterioles and right ventricle hypertrophy in the rat monocrotaline model can be prevented or reversed by POM. Previous studies have shown pulmonary vessel remodeling in MCT-treated rats and ends in the development of PH (21). Severe PH developed in MCT-injected rats within 3 weeks where the right ventricle systolic pressure is almost doubled and the RV/LV+S ratio increases by 70% compared with control animals (22). Thus, we used this rat MCT-PH model to study the changes seen in the lungs and the heart.

In the rat MCT-PH model that we used, we did not notice any plexiform-like lesion or concentric neointimal lesion that is usually seen in patients with severe PH or the SUGEN/hypoxia-induced experimental severe HP (23). Although this model does not exactly mimic the pathophysiology and pulmonary arteriopathy of human PH, many hallmarks of this disease can be seen in this model like the thickening of blood vessels wall and right ventricular hypertrophy. Moreover, the severity of arterioles remodeling in this model is higher than the other models and can serve as a good tool in screening and investigating the pharmacological effects of potential drugs on the PH (6). Rat model of PH induced by monocrotaline is superior to mice model since it showed significant increase in pulmonary pressure, right ventricular hypertrophy and remodeling of pulmonary arteries (24). Nevertheless, Rat MCT model of PH has some limitation like the lack of certain vascular pathological lesion seen in human (23). Compared with

other model of PH used in research like chronic hypoxia and hypoxia combined with Sugen-5416, MCT rat model of PH is reproducible and inexpensive, and does not require meticulous technical skills (25).

In agreement with that, our study focused on the effects of POM intake on the parameters we studied. We have shown that giving POM from day 1 after the MCT injection and for 3 weeks significantly prevented the pulmonary vascular remodeling caused by MCT and improved the pathohistological picture of the lungs. The thickening of the blood vessels walls and the widening of the tunica media of pulmonary arteries were decreased significantly by the intake of the POM juice. As well, the thickening of the alveolar walls was improved. This later improvement in the lungs histopathology was reflected on the right ventricle where the Fulton index, a parameter for the degree of right ventricle hypertrophy, was reduced significantly.

On the other hand, giving POM after the establishment of the PH (3 weeks after the MCT injection) for the same period of 21 days (from day 21 to day 42) was not accompanied by a significant improvement in the lungs' histology or the right ventricular hypertrophy. This is possibly due to the establishment of the structural changes (pulmonary vascular remodeling) that have been already formed and reached an irreversible stage (fixed components of PH), and therefore, POM was not able to affect this remodeling process. However, giving POM to those animals with irreversible remodeling was able to improve their survival rate where all rats in this group survived. In agreement with that, a previous study using the same model of HP showed that all rats without treatment have died by day 30, due to right ventricular failure, after 60 mg/kg MCT injection (26). It seems that POM administration halts or slows the progress of PH caused by the MCT (although may not reach a statistically significant difference) or POM may possess or enhance some other cardiac protective properties that are not necessarily directly related to the PH pathology (27). Moreover, this protective effects of POM could be due to the improvement in some of the hemodynamic indicators of the pulmonary circulation by punicalagin, the main phytochemicals found in POM, which causes vasodilatation through enhancement of NO-cGMP pathway in the pulmonary vascular tree (15). In addition, many pieces of evidence from human studies showed the advantageous effects of POM on the inflammation, blood circulation, muscular damage, reducing exhaustion, vasoprotective mechanisms, and the circulation levels of active blood components (28).

As previously mentioned, accumulating data shows that pomegranate has many beneficial effects on human health, and the interest in pomegranate and its health properties have been greatly increased. Pomegranate juice is rich in polyphenols like tannins and flavonoids where both have high antioxidant and anti-inflammatory activities (14, 29). Moreover, several researchers have shown that pomegranate has the highest antioxidant activities compared with other juices (14). xAlso, in vitro studies using cultured vascular endothelial cells found that POM has many effects on nitric oxide (NO) levels which acts as a potent vasodilator. POM causes more expression of endothelial nitric oxide synthase (30), protection the generated NO against oxidative destruction by superoxide anions (31) and reverts down-regulation of the expression of eNOS (32). All these effects of POM may lead to increased bioavailability of NO in lung tissues. One of the main factors implicated in the pathophysiology of PH is the imbalance between vasoconstriction and vasodilatation of the pulmonary vascular tree, and therefore the benefit of POM was possibly restoration of this balance by increasing bioavailability of NO. The improvement in the lung histology by POM could also be attributed to the inhibitory effect of NO on vascular smooth muscle cell proliferation (31). This was documented in animal studies where POM intake decelerated the progression of atherosclerosis in mice (33) and pigs, and it could reduce coronary endothelial dysfunction induced by

hyperlipidemia (29). In humans, it attenuates the platelet aggregation by reduction of thromboxane A2 production and hydrogen peroxide production (34). All these mechanisms may contribute to the direct or indirect effect of POM in reducing vascular and right ventricular remodeling and in prolonging the survival rates of treated animals.

The results of this work will hopefully broaden our understanding of the pathophysiology of pulmonary hypertension (PH) disease through the segregated analysis of the effects of pomegranate extract on a rat model of monocrotaline-induced PH. As well, it may pave the way for more useful utilization of the pomegranate extracts in the treatment of PH. Confirmation of our working hypothesis may provide a novel mechanistic foundation for an effective, economical, and safe oral therapeutic approach for PH.

Conflict of interest:

The authors declare that they have no competing interests.

Acknowledgments:

This project was funded by the scientific research fund (SRF) at the ministry of higher education, Jordan (Grant No. MPH/2/12/2015).

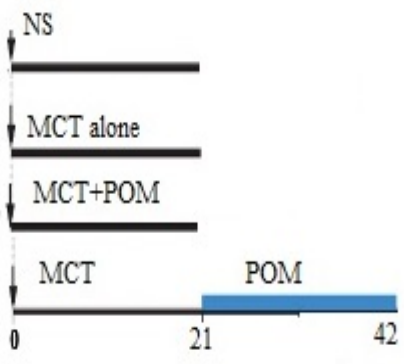

Figure 1: A diagram for the experimental protocol: male rats were injected (SC) with monocrotaline (MCT) or normal saline (PBS) on Day 0. The thick horizontal lines represent the study period for each experimental group. On Day 21, animals were either killed (control, MCT, and MCT+POM), or the MCT/POM group received POM only from Day 21 to Day 42.

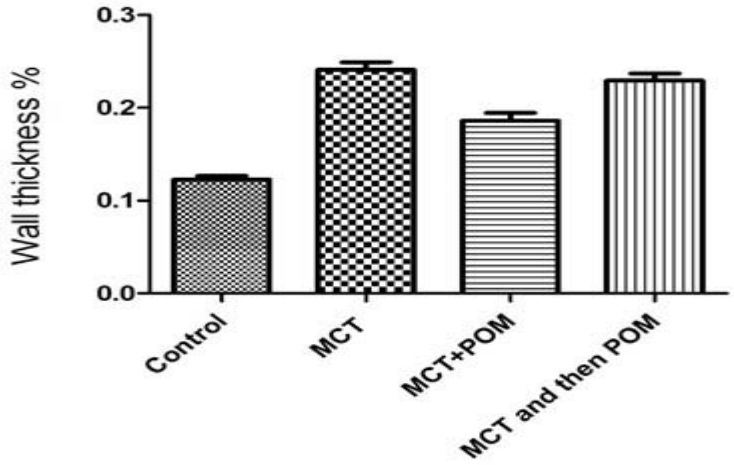

Figure 2: Pulmonary artery thickness% was increased significantly ($P < 0.05$) by all treated groups (MCT, MCT+POM, and MCT then POM). MCT+POM decreased significantly ($P < 0.05$) the thickness compared with MCT groups but MCT then POM treatment did not decrease the thickness compared with the MCT group.

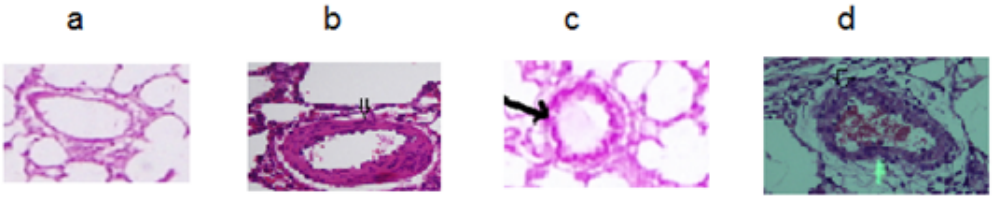

Figure 3: Representative images of H&E staining of the pulmonary arteries. The original magnification of the images was ×100 **a**. control, **b**. 21 Days after challenge in MCT, **c**. 21 Days after challenge in MCT and POM, **d**. 21 Days after challenge in MCT and then another 21 days with POM. Monocrotaline-treated animals (b) exhibit pulmonary vascular media hypertrophy and mononuclear cell infiltration compared with control animals (a).

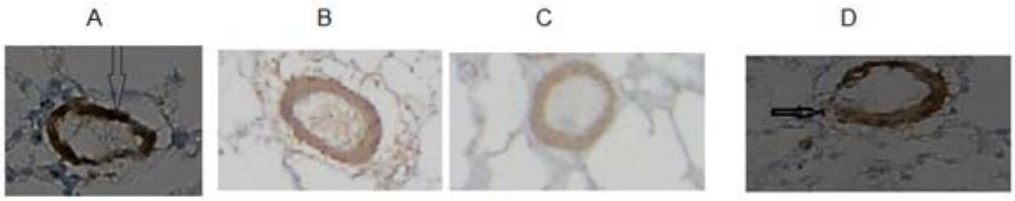

Figure 4: Immunoperoxidase labeling of pulmonary arterioles stained with anti– α smooth muscle actin antibody (brown). **A-D** representative images of paraffin sections of pulmonary arterioles stained with anti-α smooth muscle actin antibody. Control (**A**), MCT (**B**), MCT+POM (**C**) and MCT then POM (**D**).

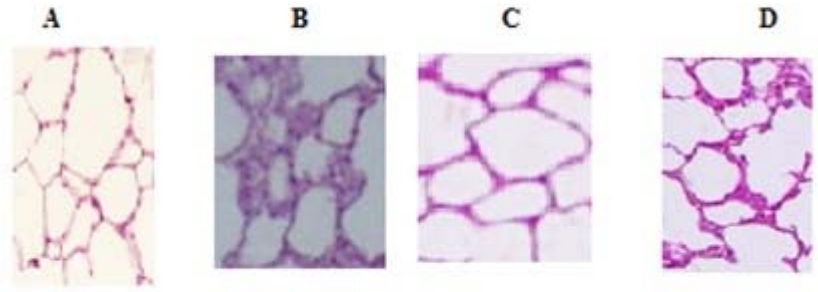

Figure 5: Pulmonary alveolar septum thickness: H&E staining of lung sections in Control (**A**), MCT alone (**B**), MCT+POM (**C**), and MCT then POM (**D**). The alveolar wall is thickened in the MCT group and this thickening was ameliorated partly in the MCT+POM group.

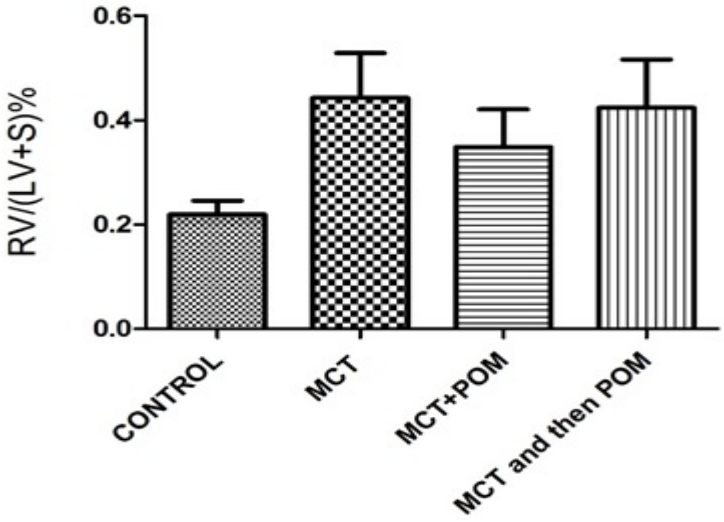

Figure 6: Measurement of the right ventricular hypertrophy: RV hypertrophy, which was measured by Fulton index [RV/ (LV + S)] of control, MCT 60 mg/kg, MCT + POM and MCT for three weeks then POM for another three weeks. All treated groups (MCT, MCT+POM and MCT then POM) significantly ($P < 0.05$) increased RV/ (LV + S) compared control group. RV/ (LV + S) is decreased significantly ($P < 0.05$) by MCT+POM treatment compared with the MCT group while MCT then POM is not significantly different from the MCT group.

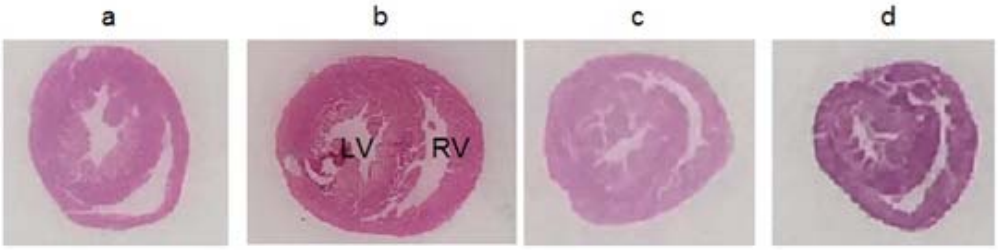

Figure 7: Hematoxylin-eosin staining for heart cross-sections in male rats. Control (**a**), MCT (**b**), MCT plus POM (**c**) MCT and then POM (**d**).

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تأثير عصير الرمان على التغيرات في الاوعية الدموية الرئوية و تضخم البطين الايمن المصاحبة لأرتفاع ضغط الدم الرئوي الناتج من المونوكروتالين -دراسة مورفولوجية

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ملخص

يرتبط ارتفاع ضغط الدم الرئوي بتغيرات هيكلية في الأوعية الدموية الرئوية. وبسبب زيادة مقاومة الأوعية الدموية الرئوية ، يتنتج تضخم البطين الأيمن وفشل القلب الأيمن. أظهرت الدراسات السابقة أن عصير الرمان له تأثيرات مضادة للالتهابات وزيادة انقسامات الخلية. كان الغرض من هذه الدراسة هو تحديد ما إذا كان تناول عصير الرمان يمكن أن يمنع أو يعكس التغيرات في الدورة الدموية الرئوية في نموذج ارتفاع ضغط الدم الرئوي في الجرذان الناجم عن المونوكروتالين. تم عمل ارتفاع ضغط الدم الرئوي في فئران ويستر عن طريق حقنة واحدة تحت الجلد من مونوكروتالين (60 مجم / كجم). كانت المجموعات التجريبية على النحو التالي: المجموعة الضابطة ، المونوكروتالين لمدة 3 أسابيع ، المونوكروتالين + عصير الرمان لمدة 3 أسابيع ، والمونوكروتالين لمدة 3 أسابيع يليها عصير الرمان لمدة 3 أسابيع (العدد = 10 لكل مجموعة). تم تحليل التغيرات النسيجية المرضية للشرايين الرئوية وتضخم البطين الأيمن الناجم عن المونوكروتالين وتأثيرعصير الرمان على هذه التغيرات. لقد وجد أن تناول عصير الرمان يؤدي الى تقليل سمك الشرايين الرئوية والبطين الأيمن بشكل كبير. :كذلك أدى إعطاء عصير الرمان مع المونوكروتالين في اليوم صفر إلى تقليل سماكة الشرايين الرئوية والحاجز السنخي وجدار البطين الأيمن. نستنتج أن تناول عصير الرمان يحسن إعادة تشكيل الأوعية الدموية الرئوية التي تم تغيرها بسبب ارتفاع ضغط الدم الرئوي الناجم عن المونوكروتالين.

الكلمات الدالة: مونوكروتالين ,عصير الرمان, تضخم البطين الأيمن, الاوعية الدموية الرئوية.

تاريخ استلام البحث 2021/3/8 وتاريخ قبوله للنشر 2021/5/21.

الناشر

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رقم الإيداع لدى دائرة المكتبة الوطنية
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المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

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

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

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

والله ولي التوفيق

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