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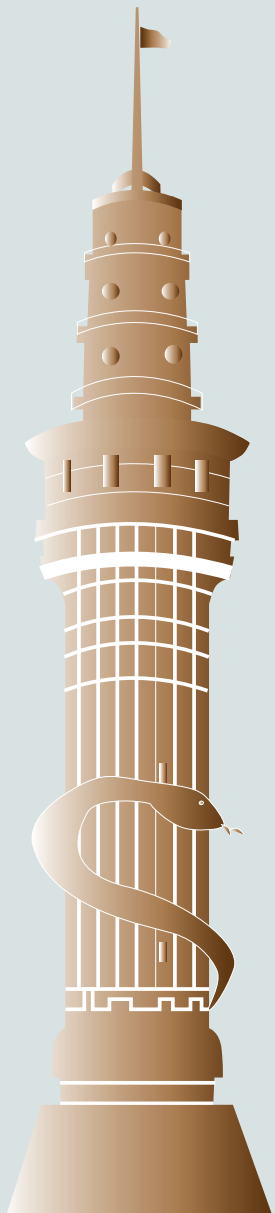

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İstanbul Journal of Pharmacy

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The manuscript should be prepared in MS Word format by using Times New Roman font (12 pt) and double-spaced on one side of the paper with adequate margins (2.5 cm).

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Units should be prepared in accordance with the International System of Units (SI).

Editorial Comments: Editorial comments aim to provide a brief critical commentary by reviewers with expertise or with high reputation in the topic of the research article published in the journal. Authors are selected and invited by the journal to provide such comments. Abstract, Keywords, and Tables, Figures, Images, and other media are not included.

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Short Papers: Please check Table 1 for the limitations for Short Papers.

Letters to the Editor: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cases, may also be submitted in the form of a "Letter to the Editor." Readers can also present their comments on the published manuscripts in the form of a "Letter to the Editor." Abstract, Keywords, and Tables, Figures, Images, and other media should not be included. The text should be unstructured. The manuscript that is being commented on must be properly cited within this manuscript.

Tables

Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

Figures and Figure Legends

Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100×100 mm). Figure legends should be listed at the end of the main document.

All acronyms, abbreviations, and symbols used in the manuscript must follow international rules and should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

For plant materials, herbarium name (or acronym), number, name and surname of the person who identified the plant materials should be indicated in the Materials and Methods section of the manuscript.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

Table 1. Limitations for each manuscript type

Type of manuscript	Word limit	Abstract word limit	Table limit	Figure limit
Original Article	3500	250 (Structured)	6	7 or total of 15 images
Review Article	5000	250	6	10 or total of 20 images
Short Paper	1000	200	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	No tables	No media

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References

While citing publications, preference should be given to the latest, most up-to-date publications. If an ahead-of-print publication is cited, the DOI number should be provided. Authors are responsible for the accuracy of references. In the main text of the manuscript, references should be cited by the author(s) surname and the publication date. When there are more than two authors, the first author should be listed followed by "et al." Please see below for examples:

One author: (Ergenç 2000)
Two authors: (Ergenç and Rollas 2000)
More than two authors: (Ergenç et al. 2000)
More than one paper in the same year by the same author (s): (Ergenç and Rollas 2000a, b)
Listed by the earliest year first for multiple citations: (Ergenç and Rollas 2000; Ergenç et al. 2001; Ergenç 2005)

The references must be listed alphabetically in the references section. The names of the journals should be written in italics and volume numbers should be indicated in bold letters. Journal titles should be abbreviated in accordance with the ISSN List of Title Word Abbreviations.

The reference styles for different types of publications are presented in the following examples.

Journal Article: Orlacchio A, Campos-Cavieres M, Pashev I, Munn EA (1979) Some kinetic and other properties of the isoenzymes of aspartate aminotransferase isolated from sheep liver. *Biochem J* **177:** 583-593.

Book Section: Benn MH, Jacyno JM (1983) The toxicology and pharmacology of diterpenoid alkaloids. In: Pelletier SW (ed./eds.) Alkaloids: Chemical and Biological Perspectives, Vol. 1, John Wiley & Sons, New York, pp. 153-210.

Books with a Single Author: Bremer K (1994) Asteraceae: Cladistics and Classification. 1st ed, Timber Press, USA.

Editor(s) as Author: Huizing EH, de Groot JAM, editors (2003) Functional reconstructive nasal surgery. Stuttgart-New York: Thieme.

Conference Proceedings: Bengisson S, Sothemin BG, (1992) Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; pp.1561-5.

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, Ferris FL 3rd; Early Treatment Diabetic Retinopathy Study Research Group. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study Kidney Int: 2004. Report No: 26.

Dissertation (Thesis): Gürdal B (2010) Ethnobotanical Study in Marmaris District (Muğla). Unpublished MSc Thesis, İstanbul University, Institute of Health Science, İstanbul.

Manuscripts Accepted for Publication, Not Published Yet: Slots J (1974) The microflora of black stain on human primary teeth. *Scand J Dent Res.*

Article by DOI: Ermut G, Karalı N, Özsoy N, Can A (2014) New spiroindolinones bearing 5-chlorobenzothiazole moiety. *J Enzyme Inhib Med Chem* doi: 10.3109/14756366.2013.800058.

Manuscripts Published in Electronic Format: (2014) World Nuclear Association. Radioisotopes in Medicine, http://www.world-nuclear. org/info/inf55.html,www.world-nuclear.org/info/inf55. html. Accessed 13.10.2014.

Treglia G, Ceriani L, Sadeghi R, Giovacchini G, Giovanella, L. (2014) Relationship between prostate-specific antigen kinetics and detection rate of radiolabelled choline PET/CT in restaging prostate cancer patients: A meta-analysis, Cli Chem Lab Med. http://www.reference-global.com/toc/cclm/current Accessed 16.09.2014.

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Accepted manuscripts are copy-edited for grammar, punctuation, and format. Once the publication process of a manuscript is completed, it is published online on the journal's webpage as an ahead-of-print publication before it is included in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.

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CONTENTS

ORIGINAL ARTICLES

İstanbul Journal of Pharmacy

OBITUARY

Professor Sevgi TATAR ULU (1969-2017)

Sevgi TATAR ULU, professor of Analytical Chemistry, passed away on July 2017 in İstanbul.

Prof. Dr. Sevgi ULU was born on 19 December 1969, in Istanbul. She completed her primary and high school education in Istanbul, at Sancaktar Hayrettin Primary School and Kocamustafapaşa High School. She graduated with academic distinction from Yildiz Technical University, Faculty of Science and Letters, Department of Chemistry in 1991. The same year she started her master's education in Analytical Chemistry Department of Faculty of Pharmacy in Istanbul University, and was appointed as research assistant in the same department in 1992.

In 1993, she completed her MS thesis titled "Spectrophotometric Determination of Fluvoxamine Maleate with 2,4,6-Trinitrobenzene Sulfonic Acid", and her PhD thesis titled "Analysis of Amlodipine Besylate in Pharmaceutical Preparations and Biological Fluids by High Performance Liquid Chromatography" in 1999. She got married to Levent Ulu in 2001, and her daughter Göksu was born in 2002.

She became Assistant Professor, Associate Professor and Professor in Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul University in 2004, 2008 and 2013, respectively. In addition to her contributions to education and teaching activities in this department, she had 53 research papers published in SCI and SCI-Expanded journals, and 7 in national journals. Her research interests were spectrophotometric, spectrofluorimetric and high performance liquid chromatographic analysis of pharmaceutical drugs. Her works received over 500 citations. She supervised 2 doctoral and 2 master's students.

Prof. Dr. Sevgi TATAR ULU attended 29 international and national congresses. In addition, she was a member of the organizing committee of 4 training seminars related to her research fields and as a member of the scientific committees in 3 congresses.

Those who had the privilege of working with Prof. Dr. Sevgi TATAR ULU, characterise her as hardworking, energetic and collaborative. She was an approachable and friendly person.

Prof. Dr. Sevgi TATAR ULU, whom we lost on 7 July 2017, continued her 25-years of academic life as an exemplary person with goodwill, diligence and dedication and she left the world by leaving a grief in our hearts. She will always be remembered.

Istanbul J Pharm 48 (3): 63-67
DOI: 10.26650/IstanbulJPharm.2018.414805
Original Article

Anticancer activities and cell death mechanisms of 1H-indole-2,3-dione 3-[N-(4 sulfamoylphenyl) thiosemicarbazone] derivatives

İdil Çetin[1], Pınar Eraslan Elma[2], Mehmet Topçul[1], Nilgün Karalı[2]*
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Cite this article as: Çetin İ, Eraslan Elma P, Topçul M, Karalı N (2018). Anticancer activities and cell death mechanisms of 1H-indole-2,3-dione 3-[N-(4 sulfamoylphenyl)thiosemicarbazone] derivatives. Istanbul J Pharm 48 (3): 63-67.

ABSTRACT

In this study, the cytotoxic effects of 1*H*-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazone derivatives namely, **4a-d** were evaluated using cell kinetic parameters including the cell index, mitotic index, labelling index and apoptotic index on HeLa cells taken from a human cervix carcinoma. All compounds were evaluated using cell index parameters at 5, 10, 20, 40, 80, 100 and 160 µM concentrations. As a result of this, it was seen that all **4a-d** compounds were effective in different concentrations. Different cell death mechanisms were proposed for **4a-d**. When all the parameters were examined, it was found that the bromine substituted **4c** was the most potent antiproliferative compound in the tested compounds. The difference was significant between the control and experimental groups ($p<0.01$). In addition, a statistically significant difference was noted among all experimental groups ($p<0.01$).

Keywords: 1*H*-indole-2,3-dione, thiosemicarbazone, antiproliferative effect, HeLa cells

INTRODUCTION

Cancer is caused by abnormal cell division and growth of certain body cells and the invasion of surrounding tissues (WHO 2018). Even though many influential chemotherapeutic agents have been approved to treat cancer, their uses are limited due to serious side effects and toxicity. Therefore, researchers have been trying to develop effective but also less deleterious new anticancer agents (Sarkar and Li 2006; Remesh 2012).

1*H*-indole-2,3-dione (isatin) is a versatile moiety and compounds bearing the isatin chemical scaffold demonstrate diverse pharmacological or biological properties (Patel et al. 2006; Zhou et al. 2006; Karalı et al. 2007; Pakravan et al. 2013; Liu et al. 2014). Furthermore, various structure activity relationship (SAR) studies have demonstrated that the presence of electron-withdrawing groups (fluor, chlor, trifluoromethoxy, nitro etc.) at the position 5 of the isatin ring scaffold enhances anticancer activity (Hall et al. 2009; Vine et al. 2009; Lv et al. 2011; Gabr et al. 2017). After approval of the 2-indolinone derivatives (unitinib, as a multi-targeted receptor, tyrosine kinase inhibitor for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor by FDA), 2-indolinone analogs have aroused interest because of their anticancer activities (Gan et al. 2009; Vine et al. 2009; Eldehna et al. 2015).

A series of 1*H*-indole-2,3-dione 3-thiosemicarbazones obtained from the condensation of substituted isatin with thiosemicarbazides has been reported as active against ovarian carcinoma, cervix carcinoma and uterine sarcomacell lines (Pape et al. 2016; Singh et al. 2017). The inhibitory effects of 1*H*-indole-2,3-dione 3-thiosemicarbazones bearing a 4-sulfamoyl phenyl moiety were investigated using human carbonic anhydrase (hCA) I, II, IX and XII isoenzymes. The tested compounds displayed selectivity against hCA IX and XII. K_I values of the compounds were found to be at low nanomolar levels (Karalı et al. 2017).

In the present study, 1*H*-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazone] derivatives were evaluated for their anticancer activities and different cell death mechanisms using cell kinetic parameters including the cell index, mitotic index, labelling index and apoptotic index.

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MATERIALS AND METHODS

Cytotoxicity

Cell Culture

The HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with FBS (Foetal Bovine Serum), streptomycin (100 µg/mL), and penicillin (100 IU/mL; Gibco).

Compound Concentrations

Stock solutions of each of **4a-d** were freshly prepared in DMSO. Seven different working solutions of each of **4a-d** (5, 10, 20, 40, 80, 100 and 160 µM) were prepared.

Real Time Cell Analyzer (RTCA): Cell Index

For this purpose, the real time cell analyzer (RTCA, xCELLigence, Roche) is used for label-free and real-time monitoring of cell properties. This system is impedance-based technology and uses specially designed microtiter plates containing interdigitated gold microelectrodes to non-invasively monitor the viability of cultured cells using electrical impedance as the measure.

In the experimental process, 100 µL of cell culture medium was added to each well for the impedance background measurement. After adding 6000 cells for each well, the final volume was 200 µL. The E-Plates (16 E-Plate) were incubated at 37°C with 5% CO_2 and monitored on the RTCA system at 15-minute time intervals for up to 24 hours without treatment of compounds and following 72 hours with treatment of compounds (Cetin and Topcul 2017).

Mitotic Index

HeLa cells were plated on coverslips and treated with either the control or experimental group for 0-72 h. The cells were then fixed using Carnoy fixative (ethanol:acetic acid, 3:1) and stained using the Feulgen method. The number of cells in the mitotic phases (including the late prophase, metaphase, anaphase and telophase; n) per total cells (3,000-3,500; C) was determined under light microscope. The MI (%) was scorred using the following formula: MI=(n/C)x100 (Topcul et al. 2013).

^{3}H-Thymidine Labelling Index

For ^{3}H-thymidine labelling index analysis (which determines cells in the S phase), HeLa cells were seeded into round coverslips which were in 24-well plates at a density of $2x10^4$ cells per well and incubated for 24 hrs. Then the cells were treated with the **4a-d** experimental concentrations. At the end of the experimental period, cells were treated with medium containing 1 µCi/mL ^{3}H-thymidine for 20 min to evaluate the labelling index (Cetin and Topcul 2017).

Autoradiography

After exposure for 3 days at 4°C, autoradiograms were developed with a D-19 developer solution (Kodak, New York, USA) and fixed with Fixaj B (Kodak, New York, USA). The coverslips were evaluated after being stained with Giemsa for 3 min. The labeling index was determined by counting at least 3000 cells/coverslip. The index is expressed as percentage labeled nuclei (Cetin and Topcul 2017).

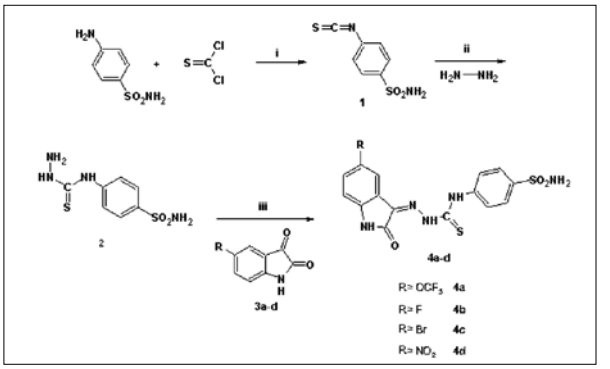
Apoptotic index (AI)

HeLa cells were collected and then fixed with methanol:Phosphate Buffered Saline (PBS) (1:1) and methanol. Fixed cells mounted on slides, stained with 0.5 mg/mL4',6-diamidino-2-phenylindole (DAPI) for 30 min and washed with PBS. Nuclear morphology of the cells was visualized using an Olympus fluorescence microscope. For evaluation of the AI, at least 250 cells were counted for control and each of the experimental groups (Cetin and Topcul 2017).

RESULTS AND DISCUSSION

Chemistry

(4-Sulfamoylphenyl)isothiocyanate **1** was prepared by reacting of sulfanilamide with thiophosgene in water containing concentrated hydrochloric acid. Hydrazine hydrate was reacted with **1** in ethanol to give N-(4-sulfamoylphenyl)thiosemicarbazide **2** 1*H*-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] **4a-d** were synthesized by reacting **2** with 5-substituted 1*H*-indole-2,3-dione **3a-d** in ethanol containing a catalytic amount of sulphuric acid (Karalı et al. 2017) (Scheme 1).



Scheme 1. Synthesis of **4a-d**. Reagents and conditions: i) H_2O, HCl, stirred ii) EtOH, stirred, cooled iii) EtOH, H_2SO_4, reflux.

Anticancer Activity

Cell Index

The cell index values obtained from the real-time cell analysis system were examined following the application of **4a-d** to HeLa cells. 5, 10, 20, 40, 80, 100 and 160 µM concentrations were used for all compounds. Figures 1-4 show the curves of the most effective concentrations of these compounds.

When the cell index values obtained following application of **4a-d** to HeLa cells were compared with the standard curve, it was shown that there were different effects for different concentrations for all compounds. The bromine substituted **4c** was tested at 5, 80 and 100 µM concentrations using the control group. **4c** showed significant DNA damage on HeLa cells at 80 and 100 µM concentrations. The trifluoromethoxy substituted **4a** and the nitro substituted **4d** were examined using the control group on HeLa cells. Both compounds had cytostatic effects as a distinct cell death type at 160 µM. The fluorine substituted **4b** showed a cytostatic effect with uncertainty at 100µM concentration to HeLa cells.

Mitotic Index

After administration of **4a** at 160 µM, **4b** at 100 µM, **4c** at 80 µM and **4d** at 60 µM for 0-72 h on HeLa cells, 3000 cells were counted for both the control and experimental groups. The mitotic index values belonging to **4a-d** are shown in Table 1.

The mitotic index is a scale for the proliferation case of a cell population. When the mitotic index values were examined, it was seen that while the bromine substituted **4c** had the most antiproliferative activity at 80 µM, the fluorine substituted **4b** had no significant effect compared with the other substances. The trifluoromethoxy substituted **4a** and the nitro substituted **4d** showed effects at 160 µM. The difference was significant between the control and experimental groups ($p<0.01$). In addition, a statistically significant difference was noted among all experimental groups ($p<0.01$).

Labelling Index

Labelling index parameters were applied at 80, 100 and 160 µM concentrations on HeLa cells for **4a-d**. 3000 cells were counted for both the control and experimental groups. Labelling index values of **4a-d** are shown in Table 2.

The effects of **4a-d** on the S phase of HeLa cells are similar to those of mitotic index values. **4c** saw the most greatly reduced DNA synthesis of HeLa cells among **4a-d**. The bromine substituted **4c** produced significant inhibition at 80 µM, while the trifluoromethoxy substituted **4a** and the nitro substituted **4d** showed an inhibitory effect at 160 µM for DNA synthesis. The difference was significant between the control and experimental groups ($p<0.01$). In addition, a statistically significant difference was noted among all experimental groups ($p<0.01$).

Apoptotic Index

In the apoptotic index parameter, 80, 100, and 160 µM concentrations were applied to **4a-d** respectively. 250 cells were counted for both the control and experimental groups. Apoptotic index values of **4a-d** are shown in Table 3.

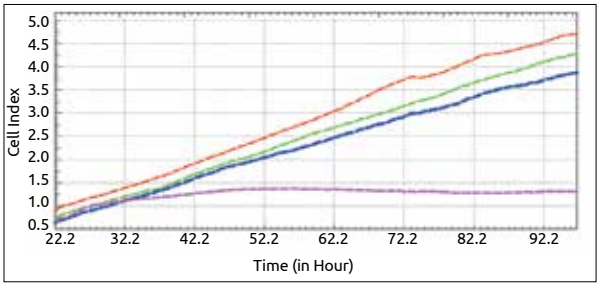


Figure 1. Cell index values of HeLa cells treated with 10, 80 and 160 µM concentrations of **4a** (--Control, -- 10µM, -- 80µM and-- 160 µM).

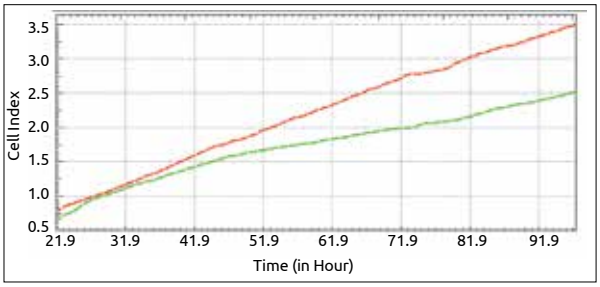


Figure 2. Cell index values of HeLa cells treated with 100 µM concentration of **4b** (--Control and -- 100µM).

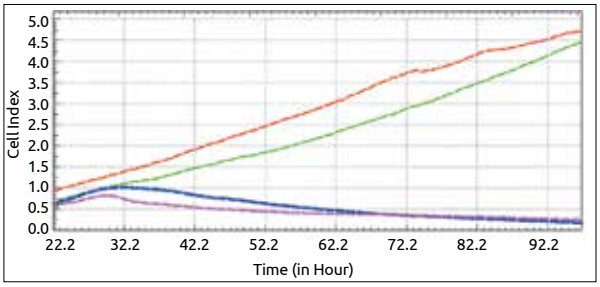


Figure 3. Cell index values of HeLa cells treated with 5, 80 and 100 µM concentrations of **4c** (--Control, -- 5µM, -- 80 µM and-- 100 µM).

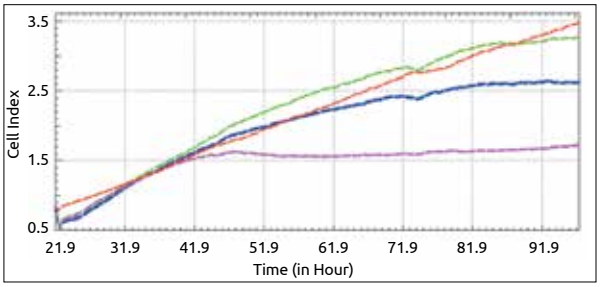


Figure 4. Cell index values of HeLa cells treated with 80, 100 and 160 µM concentrations of **4d** (--Control, -- 80 µM, -- 100 µM and-- 160 µM).

Table 1. Mitotic index (%) values of 4a-d on HeLa cells

		Mitotic Index (%)			
		4a	4b	4c	4d
Time (Hours)	Control	160 µM*	100 µM*	80 µM*	160 µM*
24	6.24±0.11^{SD}	3.56±0.03	4.15±0.07	2.94±0.04	5.18±0.06
48	8.27±0.07	3.18±0.01	4.72±0.05	1.18±0.01	4.15±0.04
72	8.96±0.05	2.96±0.03	5.14±0.02	0.38±0.02	4.13±0.05

* Significantly different $p<0.01$
SD: Standard deviation

Table 2. Labelling index (%) values of 4a-d on HeLa cells

		Labelling Index (%)			
		4a	4b	4c	4d
Time (Hours)	Control	160 µM*	100 µM*	80 µM*	160 µM*
24	5.13±0.06^{SD}	2.24±0.02	3.21±0.03	1.97±0.03	3.95±0.04
48	6.17±0.03	2.11±0.03	3.12±0.02	1.16±0.01	3.17±0.04
72	6.96±0.07	1.03±0.01	4.14±0.03	0.21±0.02	2.96±0.02

* Significantly different p<0.01
SD: Standard deviation

Table 3. Apoptotic index (%) values of 4a-d on HeLa cells

		Apoptotic Index (%)			
		4a	4b	4c	4d
Time (Hours)	Control	160 µM*	100 µM*	80 µM*	160 µM*
24	6.17±0.06^{SD}	12.15±0.02	8.16±0.03	18.19±0.11	10.23±0.06
48	7.21±0.05	12.19±0.03	9.18±0.04	19.01±0.08	14.06±0.03
72	7.96 ±0.09	14.33±0.01	10.12±0.03	22.15±0.03	15.04±0.07

* Significantly different p<0.01
SD: Standard deviation

As in other parameters, the apoptotic index values of the bromine substituted **4c** were shown to significantly increase apoptotic cell ratio at 80 µM. The trifluoromethoxy substituted **4a** and the nitro substituted **4d** were found to increase the apoptotic index values at 160 µM. The fluorine substituted **4b** had no significant apoptotic effect compared with the other substances. The difference was significant between the control and experimental groups (p<0.01). In addition, a statistically significant difference was noted among all experimental groups (p<0.01).

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Conflict of Interest: The authors have no conflict of interest to declare.

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Original Article

Ceragenins exhibiting promising antimicrobial activity against various multidrug resistant Gram negative bacteria

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ABSTRACT

Ceragenins are novel promising agents for the treatment of infections caused by multi-drug resistant microorganisms. Since colistin resistance has become a worldwide problem, the need for new treatment agents has been increasing steadily. Therefore, this study aimed to investigate *in vitro* antimicrobial activities of ceragenins (Cationic Steroid Antibiotics) (CSA-8, CSA-13, CSA-142 and CSA-192) against multidrug resistant Gram negative isolates from Turkey. Experiments were performed by using broth microdilution method against *Klebsiella pneumoniae, Morganella morganii, Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* isolates. All microorganisms except for three isolates were identified as multidrug resistant. Among tested ceragenins, CSA-13 showed the best results (MIC: 8-64 µg/ml). Nevertheless, the antimicrobial activity of CSA-8 was not significant. In conclusion, ceragenins appear to be a good candidate as antimicrobial therapy in the presence of multidrug (including colistin) resistant microorganisms.

Keywords: Ceragenins, colistin resistance, gram negative pathogens

INTRODUCTION

Antibiotic resistance is defined as the loss of activity of antibiotics against microorganisms. Antibiotic resistant bacteria, especially Gram negative bacteria, cause an increasing concern and antibiotic options used in the treatment of infectious diseases have been gradually decreasing (Zaman et al. 2017). For a long time now, colistin has been used by clinicians as only option to treat these infections. However, colistin resistance has been reported due to its widespread use in many countries. (Marchaim et al. 2011; Ah et al. 2014; Rojas et al. 2016; Sonnevend et al. 2016; Otter et al. 2017; Rossi et al. 2017). Therefore, discovering novel antimicrobials are essential in continuing to fight against antibiotic resistant bacteria.

Some of the new generation of promising agents are antimicrobial peptides (AMPs) (Döşler 2017) and ceragenins (Lai et al. 2008; Bolla et al. 2011). AMPs display broad-spectrum antimicrobial activities by interacting with the bacterial cell membrane. On the other hand, the clinical use of AMPs is problematic due to the difficulty of its synthesis and the lack of stability. Furthermore, AMP resistance may occur through some mechanisms such as secretion of proteases, release of AMP degrading enzymes, active efflux (Band and Weiss 2014; Döşler 2017). Ceragenins, Cationic Steroid Antibiotics (CSA), which are synthetic cholic acid derivatives, have the same mechanism of action as natural AMPs and mimic the activities of AMPs (Lai et al. 2008). Ceragenins can be prepared in large quantities because of their simple structures. Additionally, they are not degraded by proteases because they do not have peptide structure. Multiple studies have indicated that ceragenins display broad-spectrum activities against both Gram positive and Gram negative bacteria including drug-resistant bacteria, biofilms, fungi and viruses (Bozkurt-Guzel et al. 2014a; Durnas et al. 2016; Olekson et al. 2017).Besides, some studies have shown that the toxicity of the ceragenins is not remarkable (Saha et al. 2008; Leszczyńska et al. 2013).

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It has been shown that ceragenins have different resistance mechanisms than colistin and can be used in colistin-resistant *Klebsiella pneumoniae* isolates (Hashemi et al. 2017). Further studies are needed to determine the efficacy of ceragenins against multi-drug resistant bacteria. Thus, we aimed in this study to determine the effects of ceragenins against some multidrug resistant (including colistin) Gram negative bacteria obtained from Medical Microbiology Laboratories of the Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Turkey and Istanbul Faculty of Medicine, Istanbul, Turkey.

MATERIAL AND METHODS

Bacterial isolates

A total of 17 clinically isolates including *K. pneumoniae* (n=9), *Morganella morganii* (n=1), *Pseudomonas aeruginosa* (n=5) and *Stenotrophomonas maltophilia* (n=2) isolated from various specimens were obtained from the Medical Microbiology Laboratories of the Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Turkey and Istanbul Faculty of Medicine, Istanbul, Turkey between 2006-2016. All isolates were identified by Vitek 2 (BioMerieux, France) or API20 NE System (BioMerieux, France). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 are used as quality control strains.

Antimicrobial agents

CSA-8, CSA-13, CSA-142 and CSA-192 were synthesized from cholic acid as previously described (Guan et al. 2000). Chemical structures of ceragenins were shown in Figure 1. Meropenem, cefotaxime, colistin and levofloxacin were obtained from manufacturer (Sigma-Aldrich). Stock solutions from dry powders were prepared at a concentration of 5120 µg/mL and stored at –80°C and used within 6 months of preparation.

Antimicrobial susceptibility testing

Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, Mich., USA) supplemented with divalent cations to a final concentration of 25 mg of Mg^{2+} and 50 mg of Ca^{2+} per liter (CAMHB) was used for *in vitro* antimicrobial activity studies and Tryptic soy agar (TSA; Difco Laboratories) were used for colony counts. Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) were determined by a microdilution method according to the International Organization for Standardization (ISO) (ISO 20776-1, 2006). Serial two-fold dilutions of antibiotics were prepared in CAMHB in the test tray. A final concentration of 5×10^5 CFU/mL of inoculum was added. The trays were incubated at 37°C for 18-20 h. The MIC value was determined as the lowest concentration of antibiotics that led no visible bacterial growth. MBCs were determined at the conclusion of the incubation period by removing two 0.01 mL samples from each well demonstrating no visible growth and plating onto TSA. The MBCs were defined as the lowest concentration of antibiotic giving at least 99.9 % (three-log) reduction of the initial inocula. All experiments were performed in duplicate.

RESULTS

According to the results, all isolates were evaluated considering EUCAST limits and most of them were found to be resistant to colistin, meropenem, cefotaxime and levofloxacin (Table 1) (EUCAST, 2018a). If an isolate is non-susceptible to at least one agent in three or more antimicrobial classes, it is defined as multidrug resistant (MDR) (Magiorakos et al. 2012). Accordingly, most of the tested isolates in this study (excluding two *K. pneumoniae* isolates and one *P. aeruginosa* isolate) were defined as MDR. Two *K. pneumoniae* isolates and one *P. aeruginosa* isolate were resistant to at least one agent in the two classes, but intermediate to meropenem (MIC: 8 µg/mL). CSAs, especially CSA-13 displayed considerable inhibitory effect (MIC: 8-64 µg/mL) against *K. pneumoniae, M. morganii*, *P. aeruginosa* and *S. maltophilia* isolates even though resistant to colistin (MIC: 16->256 mL) (Table 2). The least effective agent was CSA-8 (MIC: >128 µg/mL) against all microorganisms. CSA-142 (MIC: 16-64 µg/mL) showed moderate activity. Antibacterial activity of CSA-192 was determined from 32 µg/

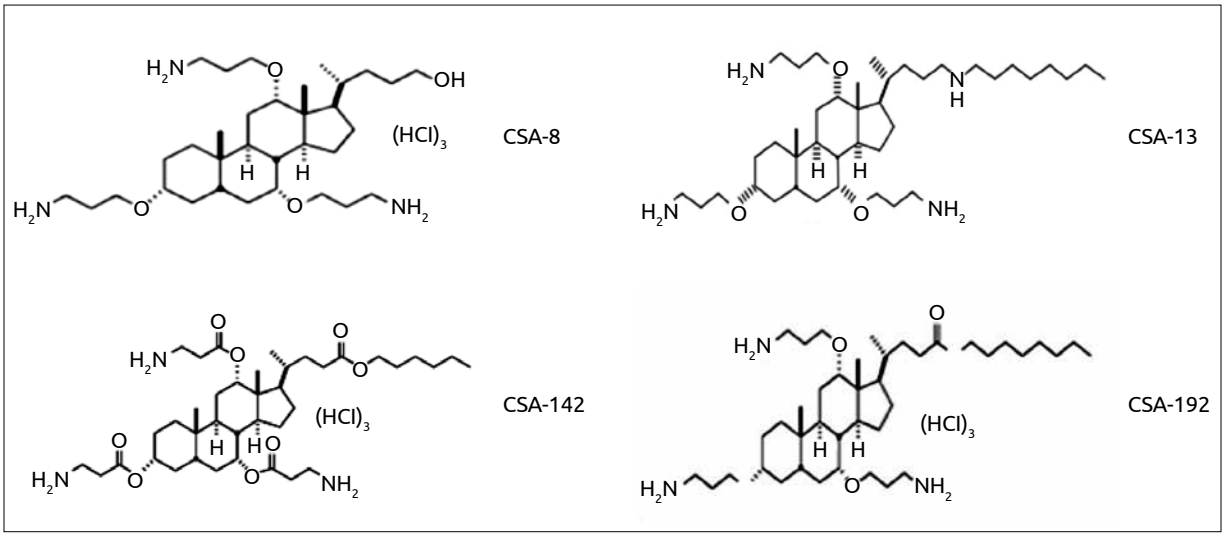

Figure 1. Chemical structures of ceragenins

mL to >128 µg/mL. Among microorganisms, all CSAs showed the lowest activity against *M. morganii* (one isolate). The MBCs were generally equal to or two fold greater than those of the MICs. All quality control results were within acceptable ranges reported in the EUCAST quality control tables (EUCAST, 2018b).

Table 1. *In vitro* antibacterial activities of antibiotics against various Gram negative bacteria

	Colistin		Meropenem		Cefotaxime		Levofloxacin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Microorganisms	µg/mL							
K. pneumoniae 1	256	256	32	128	>128	>128	8	8
K. pneumoniae 2	>256	>256	8	8	>128	>128	8	8
K. pneumoniae 3	32	32	32	32	>128	>128	16	16
K. pneumoniae 4	32	32	32	64	>128	>128	32	32
K. pneumoniae 5	32	32	16	16	>128	>128	32	32
K. pneumoniae 6	>256	>256	64	64	>128	>128	32	64
K. pneumoniae 7	32	32	8	8	>128	>128	32	32
K. pneumoniae 8	32	64	16	16	>128	>128	16	16
K. pneumoniae 9	64	64	64	128	>128	>128	32	32
M. morganii	>256	>256	16	16	128	128	16	32
P. aeruginosa 1	32	32	32	32	128	128	128	128
P. aeruginosa 2	16	16	32	32	>128	>128	8	8
P. aeruginosa 3	32	32	16	32	>128	>128	32	64
P. aeruginosa 4	32	32	16	32	>128	>128	>128	>128
P. *aeruginosa* 5	32	32	8	16	>128	>128	64	64
S. maltophilia 1	128	>128	>128	>128	>128	>128	16	64
S. maltophilia 2	32	32	128	128	>128	>128	16	32

MIC: Minimum inhibitory concentrations, MBC: Minimum bactericidal concentrations

Table 2. *In vitro* antibacterial activities of ceragenins against various Gram negative bacteria

	CSA-8		CSA-13		CSA-142		CSA-192	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Microorganisms	µg/mL							
K. pneumoniae 1	>128	>128	8	16	64	64	64	64
K. pneumoniae 2	>128	>128	16	16	64	64	64	64
K. pneumoniae 3	>128	>128	8	8	64	64	64	64
K. pneumoniae 4	>128	>128	16	32	32	32	128	128
K. pneumoniae 5	>128	>128	16	16	32	32	64	64
K. pneumoniae 6	>128	>128	16	16	32	32	128	128
K. pneumoniae 7	>128	>128	8	8	64	64	64	64
K. pneumoniae 8	>128	>128	16	16	16	16	64	64
K. pneumoniae 9	>128	>128	16	16	32	64	128	*128*
M. morganii	>128	>128	64	64	64	64	128	128
P. aeruginosa 1	>128	>128	16	16	32	32	128	>128
P. aeruginosa 2	>128	>128	8	8	32	32	32	32
P. aeruginosa 3	>128	>128	16	16	32	32	128	128
P. aeruginosa 4	>128	>128	16	32	32	32	128	128
P. aeruginosa 5	>128	>128	16	32	32	64	128	128
S. maltophilia 1	>128	>128	16	16	64	64	64	64
S. maltophilia 2	>128	>128	64	64	64	128	64	128

MIC: Minimum inhibitory concentrations, MBC: Minimum bactericidal concentrations

DISCUSSION

The emergence of MDR microorganisms, which are not affected by most of the drugs, is a growing health threat all over the world. The World Health Organization has published a list of priority pathogens resistant to antimicrobials in order to assist in the research and development of new and effective antibacterial agents. One of the most important outputs of the report is that researchers should be focused on the discovery and development of active new antibiotics against multi-drug resistant Gram negative bacterial strains. According to this report, carbapenem-resistant *P. aeruginosa, K. pneumoniae, M. morganii* are among the pathogens against which urgent precautions should be taken (WHO, 2017). Therefore, our study involves multi-drug resistant Gram negative microorganisms, including carbapenem-resistant *P. aeruginosa, K. pneumoniae, M. morganii*.

As reported previously, ceragenins have potent antimicrobial activity (Bozkurt-Güzel et al. 2014a; Bozkurt-Guzel et al. 2014b;. 2014b; Durnas et al. 2016; Olekson et al. 2017). Hashemi et al. (2017) found that CSA-13 (MIC: 2-6 µg/mL) and CSA-142 (MIC: 2-16 µg/mL) had significant antimicrobial effects and CSA-44 and CSA-131 were bactericidal against colistin resistant *K. pneumoniae* strains. Furthermore, it was also reported that ceragenins (CSA-44 and CSA-131) retained bactericidal activity against colistin-resistant bacteria. Similarly, the data shown here established that CSA-13 and CSA-142 had higher activity than other ceragenins against colistin resistant *K. pneumoniae* strains and CSA-13 (MIC: 8-16 µg/mL) had lower MIC value than CSA-142 (MIC: 16-64 µg/mL).

In the study performed by Vila-Farrés et al. (2015) regarding the activity of ceragenins against *P. aeruginosa*, it was determined that CSA-13 had the same MIC values against both colistin resistant and non-resistant strains. Chin et al. (2008) showed that CSA-13 MIC_{50} was 16 µg/mL and had potential synergistic activity against multi-drug resistant *P. aeruginosa*. In this study, CSA-13 showed similar activity (MIC: 8-16 µg/mL) against colistin resistant *P. aeruginosa* strains. According to these results, it was concluded that colistin resistance did not alter the effect of ceragenins. In another study, erythromycin-ceragenin combination against multi-drug resistant pathogens was investigated and it was shown that CSA-13 increased the activity of erythromycin by depolarizing the bacterial outer membrane. Moreover, the toxicity of CSA-13 was determined to be insignificant (Saha et al. 2008). Our study also demonstrated high antimicrobial activity of CSA-13 against multi-drug resistant *P. aeruginosa*.

To our knowledge, there is no study evaluating the activities of ceragenins against *M. morganii* and *S. maltophilia*. The study mentioned herein showed that the highest MIC value of the tested ceragenins was against *M. morganii* strain (MIC: 64->128 µg/mL). Outer membranes of these microorganisms can be a barrier for ceragenins and therefore they do not have low MIC values (Pollard et al. 2012).

According the data in Table 2, CSA-8 was found to be the least active agent. Previous studies also have shown that CSA-8 was more effective against Gram positive bacteria than Gram negative bacteria and fungi (Bozkurt-Güzel et al. 2014a; Bozkurt-Guzel et al. 2014b). The low activity of CSA-8 may be due to the lack of the hydrophobic side chain and being more hydrophilic than the other compounds.

Consequently, in the present study, multiple drug resistance was detected in most of the tested bacteria. Apparently, new agents are needed to treat the diseases caused by these bacteria. Our study showed that ceragenins, especially CSA-13, are promising agents against colistin-resistant Gram negative strains. However, additional studies are also needed to determine activity and safety of ceragenins.

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Original Article

Alkaloid profiling in *Galanthus gracilis* Celak. from western Turkey by GC/MS

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ABSTRACT

Galanthus L. is generally acknowledged as a valuable biological source due to its Amaryllidaceae alkaloids with unusual chemical structures and a wide variety of biological activities. Among these alkaloids, galanthamine is the first example of their practical application in medicine as a potent and selective inhibitor of the enzyme acetylcholinesterase, and already present on the market as a therapeutic agent for Alzheimer's disease. Lycorine is the main Amaryllidaceae alkaloid deeply investigated for its biological activity for many years. In this study the alkaloidal profile of *G. gracilis* collected from western Aegean (Alankıyı/Bayındır) will be illustrated for the first time. GC-MS profiling was carried out on the crude alkaloidal extract obtained from dried and powdered plant material. 11 alkaloids were detected by GC-MS, including graciline, demethylhomolycorine and tazettine as the major ones.

Keywords: *Galanthus gracilis*, Amaryllidaceae, graciline, demethylhomolycorine, tazettine

INTRODUCTION

Amaryllidaceae is a monocotyledon family which is formed by about 85 genera and 1100 species comprising mostly tropical or subtropical plants. *Galanthus* L. is a very interesting genus belonging to the family Amaryllidaceae considered as a very large family with 85 genera and 1100 species (Evans et al. 2002). The genus *Galanthus* L. (Amaryllidaceae) is represented by 14 species (15 taxa) in Turkey (Davis 2006). This genus has been very well known as producers of numerous alkaloids with interesting chemical structures and biological activities such as antitumor, antiviral and acetylcholinesterase inhibitory activity. Previous reports on the alkaloids of this genus reveal that *Galanthus* species possess Amaryllidaceae alkaloids with potentials to be models for new synthetic therapeutic compounds. The most reputed Amaryllidaceae alkaloid galanthamine is a drug prescribed for Alzheimer's disease and it is widely used all around the world (Gabrielsen et al. 1992; Lopez et al. 2002).

Within the course of some ongoing phytochemical studies on Turkish *Galanthus* taxa, *G. gracilis* Celak.has been collected from different localities and afforded number of new and known alkaloids. The most remarkable results of these researches were the presence of a unique subgroup of the Amaryllidaceae alkaloids called gracilines and the isolation of an unusual pentacyclic dinitrogenous alkaloid called gracilamine (Noyan et al. 1998; Unver et al. 1999; UnverKaya 2005; Bozkurt-Sarıkaya et al. 2014). Based on previous findings, *G. gracilis* is a valuable source for alkaloids with interesting chemical structures and a wide range of bioactivities. In the present study, we aimed to investigate the alkaloidal composition of *G. gracilis* collected from a different localisation considering the possible differences depending on climatic and geographical factors. The results have been compared with the ones of previous findings.

This study was presented at the "63. International Congress and Annual Meeting of the Society of Medicinal Plants and Natural Product Research-GA 2015 ", "23-27 August 2015", "Budapest, Hungary".

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MATERIAL AND METHODS

Plant material

G. gracilis was collected from Alankıyı/Bayındır in the province of İzmir and identified by Prof. Dr. Mustafa Ali Onur from Ege University, Faculty of Pharmacy, Department of Pharmacognosy.

Extraction

The extract was prepared from the bulbs of *G. gracilis*. Briefly, air-dried and powdered plant material (500 mg) was extracted three times using methanol (5 mL) in an ultrasonic bath for 30 minutes at room temperature. After the extraction procedure, solvent was evaporated under *vacuo*. The residue was dissolved in 10 mL 2% H_2SO_4, and the neutral compounds were removed with petroleum ether (3 ×10 mL). The acidic aqueous phases were basified with 25% NH_4OH up to pH 9–10 and extracted with $CHCl_3$ (3×10 mL). The combined chloroform extracts were then dried over anhydrous Na_2SO_4 and filtered through filter paper, and the organic solvent was distilled under *vacuo* to obtain the alkaloidal extract. The extract was dissolved in methanol (1 mg extract in 500 µL CH_3OH) prior to GC/MS Analysis.

GC-MS analysis

Thermo GC-Trace Ultra Ver: 2.0., Thermo MS DSQ II (Thermo Fisher Scientific, San Jose, CA, USA) was used to carry out GC-MS analysis in the electron impact mode (EI, 70 eV).

The oven temperature was programmed as: 80˚C for 1 min, 80-250˚C (10 ˚C X min^{-1}), 250˚C for 2 min, 250-300˚C (10 ˚C X min^{-1}) and 300˚C for 10 min. The injections were run at 250˚C, in the splitless mode. A TR-5 MS column (30 m × 0.25 mm × 0.25 µm) and helium (at a flow rate of 0.8 mL min^{-1}) were used as a stationary phase and carrier gas respectively.1 mg extract was dissolved in 500 µL methanol. Mass spectra of chromatographic peaks were analysed and evaluated using software Xcalibur (version 2.07; Thermo Fisher Scientific San Jose, CA, USA). Identification of the compounds were followed out checking the mass spectral fragmentations against the standard reference spectra from NIST MS Search 2.0 (National Institute of Standards and Technology, Gaithersburg, MD, USA) or the spectra of the authentic standards (S) which were previously isolated by our working team or spectral data procured from the literature. The percentage of the total ion current (TIC) for all the compounds were calculated and given as can be seen in Table 1. The area under the GC-MS peaks points out not only the concentration but also the intensity of mass spectral fragmentation of detected compounds. The ratio of each compound in the extract was exhibited as the percentage of the total alkaloid content in Table 1 and Figure 1.

The area of the GC-MS peaks depends not only on the concentration of the related compounds but also on the intensity of their mass spectral fragmentation.

RESULTS AND DISCUSSION

As previous reports shows, *Galanthus gracilis* collected from Yılanlıdağ/Muğla is hosting plant for 20 alkaloids of 7 Amaryllidaceae skeleton types which are phenanthridine, homolycorine, galanthamine, crinine, indole, tazettine and lycorine types. Also these previous findings show that; homolycorine and 8-O-demethylhomolycorine are the main alkaloids for the aerial parts and bulbs of the plant respectively (Bozkurt-Sarıkaya et al. 2014).

Table 1. Chemical composition of the *G. gracilis* bulb methanolic extracts on GC/MS analysis (collected from Alankıyı/Bayındır).

Compound Name	Retention Time (min)	[M+]	m/z (rel.%)	Bulbs (%)
Graciline type alkaloid-1	13.29	-	225 (100), 167 (10), 139 (31), 84 (7)	0.63
Graciline (Sarıkaya et al. 2013)	15.26	283 (10)	282 (7), 264 (4),254 (10),240 (8), 227 (6),226 (21), 225 (100), 139 (7)	73.2
Graciline type alkaloid-2	15.85	-	283 (84), 268 (25), 254 (43), 240 (78), 225 (100), 215 (35), 197 (29), 139 (45)	1.63
Graciline type alkaloid-3	16.32	-	283 (100), 254 (26), 242 (38), 240 (23), 228 (68), 225 (23), 139 (23)	0.49
Galanthindole (Bozkurt-Sarikaya et al. 2014)	16.68	281 (100)	264 (15), 263 (20), 262 (22), 252 (16), 204 (10), 191 (17), 132 (15), 107 (12)	4.98
6-O-Methylpretazettine (Bozkurt-Sarikaya et al. 2014)	17.31	345 (14)	330 (16), 261 (100), 239 (29), 230 (15), 201 (21)	0.9
6-O-Methylpretazettine isomer (Bozkurt-Sarikaya et al. 2014)	17.46	345 (10)	330 (12), 261 (100), 239 (24), 230 (19), 201 (25)	<0.01
Tazettine (Berkov et al. 2009)	17.77	331 (14)	316 (10), 298 (17), 247 (100), 227 (13), 211 (13), 201 (19), 181 (19), 152 (13), 115 (17)	5.76
Hippamine (Berkov et al. 2008)	18.33	301 (80)	300 (9), 227 (90), 226 (100), 227 (15)	2.41
Homolycorine (Bozkurt-Sarikaya et al. 2014)	18.60	315 (-)	109 (100), 108 (22), 94 (3)	2.76
Demethylhomolycorine (Bozkurt-Sarikaya et al. 2014)	19.24	301 (-)	109 (100), 108 (2), 94(2)	7.24

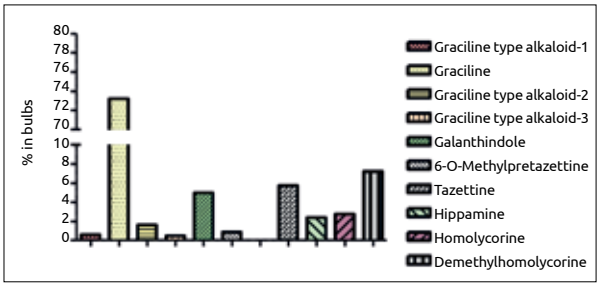

Figure 1. Graphical presentation of total amount percent of each of alkaloids in bulbs for *Galanthus gracilis*

GC/MS analysis on the bulbs methanolic extract of *G. gracilis* collected from Alankıyı/İzmir revealed that, this plant is a good source of bioactive compounds with a total of 11 alkaloids as shown in Table 1. The detected alkaloids possessed different skeleton types such as; graciline, indole, tazettine, lycorine and homolycorine. Generally, graciline, homolycorine and tazettine type alkaloids are major alkaloids in the tested material (Table 1). The extract of the bulbs of *G. gracilis* contain graciline as the main alkaloid (73.2%). Similarly, this extract comprises demethylhomolycorine (7.24%) and tazettine (5.76%) in considerable percentage. In addition an indole type alkaloid, galanthindole, is also found in significant rate (4.98%).

Aside from *Galanthus gracilis*; *G. elwesii* Hook., *G. xvalentinei*, *G. woronowi* Losinsk., *G. rizehensis* and *G. reginae-olgae* subsp. *vernalis* were already investigated by GC-MS (Berkov et al. 2004; Bozkurt et al. 2017; Conforti et al. 2010; Sarıkaya et al. 2013; Sarikaya et al. 2013). In terms of alkaloidal content, only *Galanthus gracilis* and *G. xvalentinei* nothosubsp. *subplicatus* are similar by the fact that they contain graciline type alkaloids. Among these interesting two species, *Galanthus gracilis* seems to have higher relative percentage of graciline types than *G. xvalentinei* nothosubsp. *subplicatus*.

CONCLUSION

It can be concluded that this plant is a valuable source of alkaloids with diverse chemical structures including graciline type alkaloids. The alkaloidal profile of *G. gracilis* naturally growing in Alankıyı/Bayındır differs from the other samples collected from Kemalpasa/Izmir and Yilanlidag/Mugla (Bozkurt-Sarıkaya et al. 2014; Sarıkaya et al. 2013). *G. gracilis* has a great value because of its extraordinary alkaloidal profile. Even this plant has completely different compounds such as graciline type alkaloids. The bioactivity of the graciline-type alkaloids is not well studied. Therefore the detection of gracilines in *G. gracilis* may also encourage chemists to synthesize these alkaloids for the purpose of investigating various bioactivities to be used in drug development studies.

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Original Article

Synthesis, characterization and biological activity studies on amide derivatives

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ABSTRACT

In this study, with the intention of finding novel anti-biofilm and antiviral agents, a series of amide derivatives were synthesized, and their structures were elucidated by FT-IR and ^{1}H-NMR and ^{13}C-NMR and MS methods. Their purity was proven by TLC, HPLC and elemental analyses. Finally, the synthesized compounds were examined for their biofilm formation and swarming motility inhibitory activities in *P. aeruginosa* PA01. These compounds were found to reduce biofilm formation by 8.7-25.6% and swarming motility by 18.3-33.8% in *P. aeruginosa* PA01 at a concentration of 200 µM. Additionally, all the compounds were evaluated in terms of their antiviral activity against influenza A viruses. The plaque inhibition assays indicated that compound 6 (*N*-(4-{[5-(ethylamino)-1,3,4-thiadiazol-2-yl]methyl}phenyl)-4-fluorobenzamide) has a considerable inhibitory effect on influenza A virus plaque formation.

Keywords: Synthesis, amide, anti-biofilm activity, swarming motility, influenza viruses, antiviral activity

INTRODUCTION

Amides are multifunctional groups found in many molecules. Not only are they used as prodrugs (e.g. salicylamide), but also they possess diverse biological activities such as anticancer (Jung et al. 2009; Xu et al. 2010; Yurttaş et al. 2014; Wang et al. 2014; Huczyński et al. 2015; Mathew et al. 2017), antimalarial (Delarue-Cochin et al. 2008; Kumar et al. 2011), insecticidal (Deng et al. 2016; Yang et al. 2016; Lv et al. 2018); antimicrobial (Huczyński et al. 2012; Soni and Soman 2014; Swapnaja et al. 2016; Wei et al. 2018), anti-inflammatory (Bai et al. 2018), antioxidant (Narender et al. 2011), antinociceptive (Czopek et al. 2016) and antithrombotic (Sashidhara et al. 2012), depending on their substituents. Moreover, amide carrying compounds were noted for their remarkable antibacterial (Mishra et al. 2008; Cui et al. 2017; Bi et al. 2018) and antifungal (Li et al. 2012; Sun et al. 2015; Yu et al. 2018) activities. It should be added that they have also attracted a great deal of attention for their significant anti-biofilm (Ballard et al. 2008; Richards et al. 2009; Rogers et al. 2010; Rane et al. 2012) and antiviral (Hao et al. 2012; Lan et al. 2017) activities.

As is known, the discovery of antibiotics made it possible to treat the infectious diseases that were once untreatable and enabled to save millions of lives by taking many dangerous bacterial infections under control. However, with the occurence of bacterial resistance and in regard to the increasing incidence of multidrug resistance in pathogenic bacteria, the identification of alternative antimicrobial drug targets to develop novel treatment strategies have become a necessity. Recently, it has been regarded that inactivating the quorum sensing (QS) system in bacteria by the use of QS inhibitors holds great promise for the treatment of infectious diseases. QS is a cell-to-cell communication system utilized by a wide variety of Gram (-) and Gram (+) bacteria to control the

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expression of virulence factors like elastase, extracellular protease, swarming, swimming motility and biofilm formation (de Kievit et al. 2000). Various types of screening have been carried out to find QS inhibitory molecules. Furanone derivatives, AHL analogs, synthetic compounds and some natural substances have been reported to possess QS inhibitory activity (Bosgelmez-Tinaz et al 2007; Galloway et al. 2011; Miandji et al. 2012).

Viruses have a simple structure and wholly depend on the host cells for almost all their vital functions. This situation makes it difficult to develop antiviral agents which are non-toxic for host cell metabolic systems. Antiviral agents that can be used against influenza A viruses (which pose great risks for human health), are also limited with NA inhibitors. These viruses belong to the *Orthomixoviridae* family and at times cause recurrent epidemics and pandemics within the global human population (Oxford 2000). Recurrent infections of influenza viruses in the human population are largely due to the continual changes occurring in the antigenic properties of virus surface glycoproteins (Laver 1984; Jimenez-Alberto et al. 2013). In particular, the changes of the viral surface antigens enable the virus to avoid the immunological defense of the host organism (Govorkova, 2000). Consequently, the control of influenza by vaccination is not completely effective. Therefore, a considerable effort is being made to develop new drugs and vaccines to combat influenza A viruses.

Hence, in this study, we synthesized a group of amide molecules with reference to p-aminophenylacetic acid and investigated their effects on biofilm formation and swarming motility in *P. aeruginosa*. Furthermore, the antiviral activities of these molecules were examined.

MATERIALS AND METHODS

Chemistry

All of the chemicals, reagents and solvents were purchased from Sigma Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Melting points were determined using Schmelzpunktbestimmer SMP II apparatus. For HPLC studies, an Agilent 1100 series system with a G1311A quaternary HPLC pump, a G1315A DAD detector, a G1379A vacuum degasser and a Kromasil 100 C18 5µm, 250 x 4.6 mm column was used. The Rt (retention time) values were determined by an isocratic HPLC grade acetonitrile/water (60:40 *v/v*) mobile phase at a flow rate of 1 ml/min with DAD detector set at 254 nm. The IR spectra were recorded on a Schimadzu FTIR 8400 S Spectrometer. The NMR spectra were recorded (in DMSO-d_6) with a Bruker spectrometer (Billerica, MA, USA) (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR, decoupled). The chemical shift values were expressed in ppm (δ scale) using tetramethylsilane as an internal standard. The mass spectral measurements were carried out by Electron Spray Ionization (ES) method on LC-MS-Agilent 1100. Elemental analysis was performed on Leco 215 CHNS-932 analyzer.

Synthesis of amide derivatives (1-6)

Firstly to obtain compound 1, p-aminophenylacetic acid (0.012 mol) was reacted with the equivalent moles of p-fluorobenzoylchloride in a chloroform media, while stirred at room temperature. Secondly, for compounds 2 and 3, the amide derivative (0.010 mol) was dissolved in concentrated sulphuric acid/methanol or ethanol media and refluxed. The precipitate was obtained through the neutralization reaction with sodium bicarbonate. Thirdly, to obtain compound 4 the methyl ester derivative was refluxed with hydrazine hydrate in an ethanol media. Fourthly, compound 5 was obtained through the reaction of hydrazide with ethyl isothiocyanate in an ethanol media. Finally, the thiosemicarbazide was reacted with concentrated sulphuric acid while stirring at room temperature for 45 minutes to obtain the compound 6 (Küçükgüzel et al. 2006; Karakuş et al. 2010). All of the compounds were purified with hot ethanol.

{4-[(4-Fluorobenzoyl)amino]phenyl}acetic acid (1): Cream solid. Yield 75%; m.p. 152 °C; MW: 273.2591 g/mol; *Rt* value: 7.69 min. FT-IR $u_{max.}$ (cm^{-1}): 3323 (O-H and N-H), 1726 (amide C=O), 1645 (carboxylic acid C=O), 1223 (Ar-F). (CAS Number: 907947-59-5).

Methyl {4-[(4-fluorobenzoyl)amino]phenyl}acetate (2): Cream solid. Yield 79%; m.p. 148 °C; MW: 287.2857 g/mol; *Rt* value: 6.00 min. FT-IR $u_{max.}$ (cm^{-1}): 3329 (N-H), 1742 (ester C=O), 1651 (amide C=O), 1221 (Ar-F). ^{1}H-NMR (DMSO-d_6/TMS) d (ppm): 3.62 (3H, s, -CH_3), 3.65 (2H, s, -CH_2-), 7.26 (2H, d, *J:* 8.40 Hz, Ar-H), 7.37 (2H, t, Ar-H), 7.68 (2H, d, *J:* 8.40 Hz, Ar-H), 8.04 (2H, t, Ar-H), 10.27 (1H, s, -CONH-). ^{13}C-NMR (DMSO-d_6/TMS) d (ppm): 39.57, 51.62, 115.12, 115.41, 120.39, 129.48, 129.60, 130.27, 130.39, 131.29, 131.33, 137.79, 162.37, 164.31 (amide C=O), 165.67, 171.67 (C=O). MS (ES m/z): 310 (M^++Na), 180, 179, 101. Elemental analysis for $C_{16}H_{14}FNO_3$ Calculated/Found (%): C: 66.89/66.28, H: 4.91/4.89, N: 4.88/4.72. (CAS Number: 2204929-37-1).

Ethyl {4-[(4-fluorobenzoyl)amino]phenyl}acetate (3): White solid. Yield 65%; m.p. 154-155 °C; MW: 301.3122 g/mol; FT-IR $u_{max.}$ (cm^{-1}): 3337, 3298 (N-H), 1722 (ester C=O), 1647 (amide C=O), 1229 (Ar-F). ^{1}H-NMR (DMSO-d_6/TMS) d (ppm): 1.19 (3H, t, -CH_2-CH_3), 3.63 (2H, s, -CH_2-), 4.05-4.12 (2H, q, -CH_2-CH_3), 7.26 (2H, d, *J:* 8.40 Hz, Ar-H), 7.34-7.40 (2H, m, Ar-H), 7.69 (2H, d, *J:* 8.40 Hz, Ar-H), 8.01-8.06 (2H, m, Ar-H), 10.26 (1H, s, -CONH-). ^{13}C-NMR (DMSO-d_6/TMS) d (ppm): 14.53, 40.26, 60.73, 115.65, 115.94, 120.89, 129.97, 130.23, 130.76, 130.88, 131.74, 131.78, 138.19, 162.87, 164.88 (amide C=O), 166.17, 171.75 (C=O). Elemental analysis for $C_{17}H_{16}FNO_3$ Calculated/Found (%): C: 67.76/68.05, H: 5.35/5.50, N: 4.65/4.53. (CAS Number: 2204959-86-2).

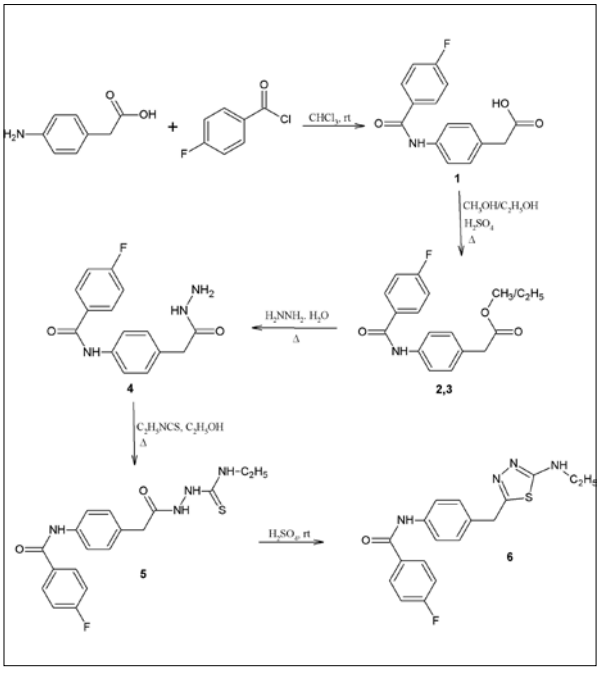
4-Fluoro-*N*-[4-(2-hydrazinyl-2-oxoethyl)phenyl]benzamide (4): White solid. Yield 86%; m.p. 352 °C (decomposed); MW: 287.2890 g/mol; *Rt* value: 2.70 min. FT-IR $u_{max.}$ (cm^{-1}): 3352, 3295, 3210 (N-H), 1645 (C=O), 1233 (Ar-F). ^{1}H-NMR (DMSO-d_6/TMS) d (ppm): 3.22 (2H, s, -CH_2-), 4.22 (2H, b.s, -NH-NH_2), 7.25 (2H, d, *J:* 8.70 Hz, Ar-H), 7.36 (2H, t, Ar-H), 7.65 (2H, d, *J:* 8.40 Hz, Ar-H), 8.02 (2H, t, Ar-H), 9.21 (1H, b.s, -NH-NH_2), 10.23 (1H, s, -CONH-). ^{13}C-NMR (DMSO-d_6/TMS) d (ppm): 39.87, 115.11, 115.40, 120.34, 129.04, 130.24, 130.36, 131.28, 131.32, 131.59, 137.17, 162.35, 164.26 (amide C=O), 165.65, 169.66 (C=O). MS (ES m/z): 310 (M^++Na), 180, 179, 101. Elemental analysis for $C_{15}H_{14}FN_3O_2$ Calculated/Found (%): C: 62.71/63.37, H: 4.91/4.97, N: 14.63/14.21. (CAS Number: 2214835-31-9).

***N*-(4-{2-[2-(ethylcarbamothioyl)hydrazinyl]-2-oxoethyl} phenyl)-4-fluorobenzamide (5):** White solid. Yield 79%; m.p. 226 °C; MW: 374.4325 g/mol; *Rt* value: 3.63 min. FT-IR $u_{max.}$ (cm^{-1}): 3314, 3196 (N-H), 1674, 1645 (C=O), 1219 (C=S), 1159 (Ar-F). ^{1}H-NMR (DMSO-d_6/TMS) d (ppm): 1.07 (3H, t, -CH_2-C$\underline{H}_2$), 3.45 (4H, s, -C$\underline{H}_2$-CH_3 and -CH_2-), 7.28 (2H, d, *J*: 8.70 Hz, Ar-H), 7.37 (2H, t, Ar-H), 7.67 (2H, d, *J*: 8.40 Hz, Ar-H); 7.93 (1H, t, N_4H), 8.01-8.06 (2H, m, Ar-H), 9.17 (1H, b.s, N_2H), 9.91 (1H, b.s, N_1H), 10.24 (1H, s, -CON$\underline{H}$-). ^{13}C-NMR (DMSO-d_6/TMS) d (ppm): 14.44, 37.13, 115.13, 115.42, 120.27, 129.40, 130.26, 130.38, 130.79, 131.27, 131.31, 137.49, 162.36, 164.27 (amide C=O), 165.66, 169.95 (C=O), 181.25 (C=S). Elemental analysis for $C_{18}H_{19}FN_4O_2S$ Calculated/Found (%): C: 57.74/58.31, H: 5.11/5.27, N: 14.96/14.94, S: 8.56/7.90.

***N*-(4-{[5-(ethylamino)-1,3,4-thiadiazol-2-yl]methyl} phenyl)-4-fluorobenzamide (6):** Light brown solid. Yield 40%; m.p. 310-311 °C; MW: 365.4248 g/mol; *Rt* value: 4.20 min. FT-IR $u_{max.}$ (cm^{-1}): 3310, 3188 (O-H and N-H), 1651 (C=O), 1231 (Ar-F), 760 (C-S-C). ^{1}H-NMR (DMSO-d_6/TMS) d (ppm): 1.13 (3H, t, -CH_2-C$\underline{H}_2$), 3.18-3.27 (2H, m, -C$\underline{H}_2$-CH_3), 4.13 (2H, s, -CH_2-), 7.27 (2H, d, *J:* 8.70 Hz, Ar-H), 7.37 (2H, t, Ar-H), 7.57 (1H, t, -NH-), 7.70 (2H, d, *J:* 8.40 Hz, Ar-H), 8.03 (2H, t, Ar-H), 10.27 (1H, s, -CON$\underline{H}$-). ^{13}C-NMR (DMSO-d_6/TMS) d (ppm): 14.24, 34.99, 115.13, 115.42, 120.61, 128.82, 130.27, 130.39, 131.27, 131.30, 133.24, 137.82, 157.09, 162.37, 164.32, 165.67, 168.78 (amide C=O), 169.69. MS (ES m/z): 357 (M^++1), 189, 182, 179, 101. Elemental analysis for $C_{18}H_{17}FN_4OS.½H_2O$ Calculated/Found (%): C: 59.16/59.34, H: 4.96/4.87, N: 15.33/15.18, S: 8.77/8.74.

Anti-biofilm activity

The anti-biofilm capacities of the substituted-amide derivatives were examined using the biofilm assay. The overnight culture of *P. aeruginosa* PA01 strain was diluted to an OD_{600} of 0.02. 1mL aliquots of the diluted cultures were allocated in polystyrene tubes and incubated at 32°C for 10 h. Nonadherent cells were removed. The biofilms were dyed with 1 ml of crystal violet (0.3%) and the absorbance was measured at 570 nm using a spectrophotometer (Truchado et al. 2009).



Scheme 1. Synthetic route of compounds **1-6.**

Swarming motility assay

The swarming motility was measured as described by Rashid et al. (2000). Five microliters of PA01 cultures were inoculated onto the surface of swarm plates containing Bacto Agar (0.5%), Nutrient Broth and glucose (1%). This was completed both in the presence and absence of the test compounds and then incubated overnight at 37°C for 24 h.

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were used for plaque inhibition assays. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (Gibco), penicillin G (100 U/mL) and streptomycin (100 µg/mL), and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. The antiviral activities of the synthesized compounds were investigated on influenza A virus, strain A/WSN/33 (H1N1). The viruses were grown in the allantoic cavity of 10 day-old chick embryos at 35.5 °C for 48 h. The allantoic fluid was clarified by centrifugation at 3,000g for 10 minutes, passed through 0.45 µm sterile filter, and the filtrate was stored in small aliquots at 80 °C.

Plaque inhibition assay

For the plaque inhibition assay, confluent monolayer cultures of MDCK cells in 12-well plate were washed twice with DMEM (-), and infected with influenza viruses at the appropriate multiplicity of infection (moi). After adsorption for 30 min at 37 °C, virus inoculums were completely removed, and the cell monolayers were overlaid with a maintenance medium (DMEM containing 0.6% agarose, 0.2% Bovine Serum Albumin and 4 mg/mL TPCK-treated trypsin). In test conditions, synthesized compounds were added to a maintenance medium at defined concentrations. The plates were incubated at 34 °C for 2-3 days, and plaques were visualized by staining the cells with amido black (Turan et al. 1996; Güveli et al 2018).

RESULT AND DISCUSSION

In the present study, six amide derivatives (compounds 1-6) were synthesized from p-aminophenylacetic acid. The synthetic route of compounds is represented in Scheme 1.

Their purity was proven by TLC, HPLC and elemental analyses. Also, their structures were elucidated by FT-IR, ^{1}H-NMR, ^{13}C-NMR and MS spectral methods. IR absorption bands due to amide C=O and aromatic C-F stretching bands were recorded at 1726-1645 and 1233- 1159 cm^{-1}, respectively. According to the ^{1}H-NMR spectra, the –CH_2- and amide N-H peaks were observed at 3.22-4.13 and 10.23-10.27 ppm as singlets, in turn. In addition, the ^{13}C-NMR spectra exhibited resonances at 164.26-168.78 assigned for amide C=O. Aside from these, the elemental analysis and MS spectral data results were in accordance with the compounds structures.

Table 1. Effect of compound 1-6 derivatives on the biofilm formation and swarming motility of *P. aeruginosa* PA01 strain. The data represents the averages from the results of three independent experiments.

	Biofilm Formation Inhibition (%)	Swarming Motility Inhibition (%)
1	13.9	28.9
2	8.7	22.4
3	25.6	18.3
4	13.0	31.4
5	19.0	32.2
6	17.3	33.8

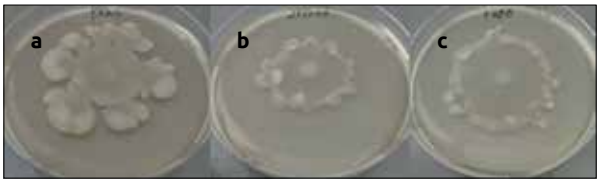


Figure 1. a-c. Representative images of inhibition of *P. aeruginosa* PA01 swarming motility. (a) Control (no compound), in the presence of compound 6 (b) and compound 2 (c).

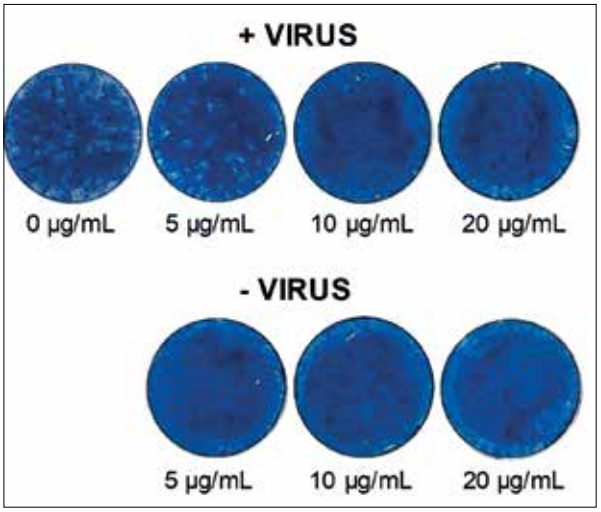


Figure 2. The influenza virus plaque formation inhibition of compound **6**. Monolayers of Madin-Darby canine kidney epithelial cells (MDCK) were infected with influenza viruses (A/WSN/33) at the appropriate moi (upper panel). After 30 min of infection exposure, virus inoculums were removed, and monolayers were overlaid with 0.6% agarose-maintenance medium, both with and without compound **6**. Lower panel shows non-infected MDCK cells monolayers treated with compound **6** at specified concentrations. After 2-3 days of incubation, monolayers were fixed and stained with amido black dye solution.

The synthesized compounds' anti-biofilm capacities were confirmed by the biofilm assay. Biofilm formation causes serious problems in both medicine and industry. Biofilm-associated bacteria are more resistant to antimicrobials than planktonic cells. We tested the effects of compounds **1-6** on biofilm formation of *P. aeruginosa* PA01. According to the results these molecules inhibited biofilm formation by 8.7-25.6% at 200 µM concentration. Among the tested compounds, compound **3** was found to be the most active one, reducing the biofilm formation by 25.6% in *P. aeruginosa* PA01 at a concentration of 200 µM. We also performed a swarming motility assay. Swarming motility plays an important role in the early stages of biofilm development and antibiotic resistance. The swarming motility of *P. aeruginosa* PAO1 was assayed both in the presence and absence of test compounds. Swarming plates were supplemented with 200 µM synthesized compounds. The treatment of *P. aeruginosa* PAO1 with these compounds resulted in reductions in swarming motility by 18.3-33.8% (Table 1, Figure 1).

The antiviral activity of compounds 1-6 were revealed by using plaque inhibition assays on influenza A viruses. Among the synthesized compounds tested on influenza virus plaque formation, Compound 6 showed an inhibitory effect (Figure 2). Plaque formation by influenza A viruses was almost completely inhibited by this compound at concentrations of 10 µg/mL. Compound 6 did not show any cytopathic effect on MDCK cells at 5-20 µg/mL (Figure 2).

Influenza viruses are enveloped viruses having a negative polarity and a segmented RNA genome. Despite the simple structure, they have multi-stage complex replication strategies. Therefore, it is difficult to reach a firm conclusion about the action mechanism of compound 6 on the influenza virus replication based on the results of the plaque inhibition assay. The research will continue to elucidate the mode of action of this molecule. Compound 6 differs from the other 5 compounds in terms of thiadiazole group. This group may therefore be considered as important for antiviral activity (Gan et al. 2017).

CONCLUSION

A series of amide derivatives (1-6) were obtained from p-aminophenyl acetic acid, characterized by several spectroscopic methods (IR, NMR, MS) and elemental analysis. They were also evaluated for their anti-biofilm and antiviral effects. The results suggested that compounds 1-6 could be used as antibiofilm agents in combination with conventional antibiotics to increase the efficiency of current antimicrobials. Also, depending on antiviral activity studies, it can be said that compound 6 has potential as an anti-influenza virus agent. Further studies are in progress.

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Conflict of Interest: The authors have no conflict of interest to declare.

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Original Article

Evaluation of biorelevant media to investigate the dissolution properties on flurbiprofen and to assess cytotoxicity effects on Caco-2 cell line

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ABSTRACT

Biorelevant media are used to simulate the physiological conditions in terms of components, pH, osmolality and buffer capacity of the human stomach and intestine in both fasted and fed states In this study, we aim to apply the biorelevant media to Caco-2 cell lines to investigate the cytotoxicity effects via the cell viability ratio and to compare the solubilizing effects of various dissolution media on a poorly soluble model drug. Flurbiprofen (Biopharmaceutics Classification System, BCS Class 2) was selected as a model drug.

In dissolution studies the pH effects were predominant at higher pH values, while bile salt effects were dominant at lower pH values. The preparation method, bile salts and the phospholipids did not show any additional effect on Caco-2 cell viability. In the cytotoxicity test, fed state media caused an additional 10-15% decrease in cell viability, compared to the fasted state. Similar results were obtained when using the blank of these media which did not include the bile salt or phospholipid. From this, it is evident that this decrease resulted from the pH values, not the components. In conclusion, the cytotoxicity assessment showed that all the biorelevant media were compatible with 70-90% of cell viability for at least 24 h, and this ratio might be increased by modifying the pH.

Keywords: Biorelevant media, flurbiprofen, cytotoxicity

INTRODUCTION

Many studies have been conducted to develop a dissolution media which better reflects the contents of the human gastrointestinal system. In those studies, researchers added some enzymes, surfactants, bile salts, phospholipids, lipolysis products etc. to the media (Dressman et al. 1998; Galia et al. 1998; Tang et al. 2001; Wiedman et al. 2002; Jantraid et al. 2008). Additionally, pH, surface tension, and the osmolality of these media were taken into consideration. Regarding the effect of food on the absorption of BCS Class 2 drugs, the dissolution media for the fed state were also developed separately (Dressman et al. 1998; Galia et al. 1998; Jantraid et al. 2008).

Health authorities have not yet approved any biorelevant media other than compendial media. However, Dressman developed two basic media for reflecting intestine in the fasted and fed states (Dressman et al. 1998). These media are known as Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF). These media have attracted the attention of a many scientists and much research has been conducted in their use (Galia et al. 1998; Nicolaides et al. 1999; Kostewicz et al. 2002; Fagerberg et al. 2010). Moreover, several other media were developed based on these media (Marquez 2004; Jantraid et al. 2008; Fatouros et al. 2009; Kleberg et al. 2010; Klein 2010; Fuchs et al. 2015; Zhou et al. 2017). When the dissolution tests that were performed using these media provided a better correlation in vivo (Mathias et al. 2015; Xu et al. 2017), researchers started to use them in permeability studies (Patel et al. 2006; Birch et al. 2018). However, certain components in these media such as bile salts and phospholipids raised suspicions regarding the possible toxic effects on cell lines (Ingels

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and Augustijns 2003; Birch et al. 2018). Apart from the components of these media, the preparation process is also important. Due to the physicochemical properties of bile salts and lecithin and the use of organic solvents in the process, the preparation of these media is not simple. To standardize the media preparation, an instant powder mix of these components was obtained (SIF Powder®) by Biorelevant.com (Boni et al. 2009).

Flurbiprofen is a rapidly absorbed, non-steroidal anti-inflammatory drug, with 96% oral bioavailability following oral administration. Absorption is increased with food (Pargal et al. 1996) and it eliminates 75-80% as metabolites and 20-25% as the unchanged drug in the urine. Flurbiprofen is a weak acid with a pK_a of 4.22. It is a BCS Class 2 drug and practically insoluble in water. It has a solubility of 0.0080 mg/mL at pH 1.2 and its solubility increases with pH (Li and Zhao 2003). Yazdanian et al. calculated the permeability (P_{app}) of flurbiprofen as higher than verapamil used as the reference drug with a P_{app} of $20.1x10^{6}$±2.7 x 10^{6} cm/s (Yazdanian et al. 2004).

In this study, we aim to evaluate the possible cytotoxic effects of a high permeable model drug when prepared conventionally and when prepared from the instant powder. For this reason, we selected a BCS Class 2 drug - flurbiprofen. Moreover, we performed solubility and dissolution tests with the commercial product containing the model drug to compare the media against themselves. In addition to the biorelevant media, pharmacopeial media and blank media not including the bile salts and phospholipids were also used to assess the pH effects.

MATERIALS AND METHODS

Sodium taurocholate 97% pure (high quality: HQ), egg-phosphatidylcholine, MTT and trypan blue were purchased from Sigma-Aldrich® (USA). All the chemicals and reagents were purchased from Merck® (Germany). SIF Powder® (Biorelevant.com, United Kingdom) is a patented formulation of sodium taurocholate and lecithin with the molar ratio of 4:1 which corresponds with Dressman's formulation (Dressman et al. 1998). The biorelevant media prepared using instant powder are defined as SIF-FaSSIF and SIF-FeSSIF in this study.

Dulbecco's Modified Eagle's Medium (DMEM), EDTA, Fetal bovine serum (FBS) and RPMI 1640 were purchased from Biochrom, Germany and Caco-2 cells were obtained from Cell Culture Collection, Turkey. Flurbiprofen (Sun Pharmaceuticals, India) was supplied from Drogsan Pharmaceuticals (Turkey). Ansaid® (Pfizer, Turkey) 100 mg film-coated tablets were purchased from a local drug market.

Preparation of dissolution media

The classical dissolution media of pH 1.2, pH 4.5, and pH 6.8 were prepared according to USP. FaSSIF and FeSSIF were prepared as previously reported (Marquez 2004). SIF-FaSSIF and SIF-FeSSIF, Blank FaSSIF, Blank FeSSIF were prepared in accordance with the protocols of Biorelevant.com. The compositions of the biorelevant media and blank media are shown in Table 1.

Solubility

Solubility measurements of the samples were performed using the shake-flask method and all the solubility experiments were conducted in triplicate. According to the method, the excess of the drug powder was added to 50 mL of different dissolution media and stirred at 37.0˚C±0.1˚C in a shaking incubator water bath. The equilibrium time was set to 24 h. The final solution was then filtered through a 0.45 µm (Millipore Millex-HV, USA) membrane filter and analyzed using a UV spectrophotometer (Shimadzu, UV-170, Japan). To evaluate the solubility results of the drug, dose number (D_0), which is defined as the ratio of drug concentration in the administered volume to the saturation solubility of the drug (Oh et al. 1993) was used. It was calculated using Equation 1.

$$D_0 = \frac{M_0/V_0}{C_S} \quad \text{Eq 1}$$

where M_o is the dose of drug administered, V_o is the administered volume, and C_s is the saturation solubility. Fluid volume used with the drug was set as 250 mL - the volume of a glass

Table 1. Compositions of the FaSSIF and FeSSIF (Dressman et al. 1998; Biorelevant.com)

Compositions	FaSSIF*	FeSSIF*	SIF-FaSSIF	SIF-FeSSIF	Blank FaSSIF	Blank FeSSIF
Sodium taurocholate	3 mM	15 mM	3 mM	15 mM	-	-
Lecithin	0.75 mM	3.75 mM	0.75 mM	3.75 mM	-	-
$NaH_2PO_4.H_2O$	1.977 g	-	1.977 g	-	1.977 g	-
Glacial acetic acid	-	8.65 g	-	8.65 g	-	8.65 g
NaCl	3.093 g	11.874 g	3.093 g	11.874 g	3.093 g	11.874 g
NaOH (pellets)	0.174 g	4.04 g	0.174 g	4.04 g	0.174 g	4.04 g
Deionized water (qs)	500 mL	1000 mL	500 mL	1000 mL	500 mL	1000 mL
pH	6.5	5.0	6.5	5.0	6.5	5.0

*Lecithin was dissolved in dichloromethane, after emulcification dichloroethane was evaporated.
FaSSIF: Fasted State Simulated Intestinal Fluid, FeSSIF: Fed State Simulated Intestinal Fluid , SIF-FaSSIF: Fasted State Simulated Intestinal Fluid prepared using instant powder, SIF-FeSSIF: Fed State Simulated Intestinal Fluid prepared using instant powder, Blank FaSSIF: Fasted State Simulated Intestinal Fluid media not including the bile salts and phospholipids, Blank FeSSIF: Fed State Simulated Intestinal Fluid media not including the bile salts and phospholipids

of water. While a D_0 which is equal to or lower than 1 means a high-solubility, a D_0 higher than 1 indicates a low-solubility.

In vitro dissolution studies

USP apparatus 2 (PharmaTest, Germany) was used for all dissolution tests. The dissolution studies were carried out at 37±0.5˚C in 900 mL of dissolution media and with a rotational speed of 50 rpm. At each predetermined sample time intervals (5, 10, 15, 20, 30, 45 and 60 minutes), 5 mL of the sample was taken, and 5 mL of blank medium was replaced. All samples were filtered using 0.45 µm (Millipore Millex-HV, USA) membrane filter and after diluting, they were analyzed by the validated UV spectrophotometric method (Shimadzu, UV-170, Japan). All experiments were performed in triplicate. The dissolution test results were evaluated using the similarity factor (f_2) to compare the mediums.

$$f_2 = 50 \cdot log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{t=1} n\,(R_t - T_t)^2\right]^{-0.5} \cdot 100\right\} \quad \text{Eq 2}$$

Where n is the number of time points, R_t is the dissolved amount of the reference at time t, and T_t is the dissolved amount of the test at time t.

Spectrofotometric analysis

A Shimadzu UV-170 Spectrophotometer (Japan) was used for UV analysis. All assay and dissolution studies were analyzed using a UV-spectrometer. The maximum absorbance values of the drugs in various media were different. The wavelengths (λ_{max}) that are being used were 246 nm (pH 4.5, pH 6.8, Blank FaSSIF, FaSSIF and Blank FeSSIF), 248 nm (SIF-FeSSIF) and 250 nm (FeSSIF and SIF-FeSSIF).

Cytotoxicity assessments

Caco-2 cells were grown at 37˚C in an atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. A confluent cell line was washed with trypsin/EDTA solution (0.05%/0.02%) and kept in an incubator for 5-10 minutes and then centrifuged. The cell line was homogenized by DMEM with 10% of serum and 1% of antibiotic. Three passages were performed. For the cell count 0.1 mL of trypan blue was added to 0.9 mL of cell suspension and the cell count was conducted by a hemacytometer. The cell viability was then checked with an optical microscope. The cells were homogenized in DMEM containing 10% FBS and 1% antibiotic and then a $4x10^4$ cell/mL cell suspension was prepared and transferred to 96-well cell plates (100 µL/well) and incubated in 5% CO_2 for 24 hours.

Table 2. Solubility and dose number values of flurbiprofen in different dissolution media.

Dissolution media	Solubility±SD (mg/mL)	D_0±SD	Dissolved % in 30 min Mean±SD
pH 4.5	0.0555±0.0003	7.21±0.04	39.6±0.4
pH 6.8	2.53±0.05	0.323±0.006	90.9±0.9
BlankFaSSIF	1.78±0.03	0.224±0.003	93.2±1.7
FaSSIF	1.25±0.02	0.158±0.003	95.7±3.0
SIF-FaSSIF	1.70±0.01	0.237±0.001	98.6±0.9
BlankFeSSIF	0.0756±0.0014	5.29±0.09	58.3±1.0
FeSSIF	0.529±0.007	0.756±0.011	76.1±2.4
SIF-FeSSIF	4.20±0.02	0.0946±0.0005	104.5±0.1

D_0: Dose number, SD: Standard deviation, FaSSIF: Fasted State Simulated Intestinal Fluid, FeSSIF: Fed State Simulated Intestinal Fluid , SIF-FaSSIF: Fasted State Simulated Intestinal Fluid prepared using instant powder, SIF-FeSSIF: Fed State Simulated Intestinal Fluid prepared using instant powder, Blank FaSSIF: Fasted State Simulated Intestinal Fluid media not including the bile salts and phospholipids, Blank FeSSIF: Fed State Simulated Intestinal Fluid media not including the bile salts and phospholipids

MTT viability test

The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] test is a colorimetric assay test that can be used to determine cell viability (mitochondrial activity) measuring the extent of formazan formation after the lysis of the living material and the solubilization of formazan crystals (Berridge et al 2005).

The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] test was performed to evaluate the cytotoxicity of sodium taurocholate, lecithin and flurbiprofen in biorelevant media with incubation times of 1 and 24 hours and concentrations of 100 µM and 500 µM. These concentrations were selected with reference to previous transport studies (Laitinen et al. 2007). The cell viability values were calculated as a percentage in control groups according to the following equation:

$$Cell\ viability\ (\%) = \frac{A_{test}}{A_{control}} x\ 100 \quad \text{Eq 3}$$

where A_{test} is the absorbance of test and $A_{control}$ is the absorbance of DMEM.

RESULTS AND DISCUSSSION

Solubility

The solubility studies were assessed following two methods: 1) the effect of pH and 2) the effect of concentrations of sodium taurocholate and lecithin. In addition, the preparation method of the biorelevant media was also considered. The solubility test results of flurbiprofen and estimated dose figures are summarized in Table 2. Flurbiprofen is a weak acid, thereby it is soluble when the pH value is greater than its pK_a value of 4.22. For this reason, flurbiprofen did not practically dissolve at pH 1.2 and the solubility increased with pH. When the solubilization effects of sodium taurocholate and lecithin were not considered, flurbiprofen showed the maximum solubility at pH 6.8, the highest pH. The solubility at pH 6.8 was four times higher than at pH 4.5. The solubility values of flurbiprofen at different pH levels (Table 2) were in a strong correlation with the literature data (Li and Zhao 2003). To evaluate the effect of sodium taurocholate and lecithin in the fasted state (Table 3), Blank FaSSIF was compared with FaSSIF and SIF-FaSSIF. The solubility values of flurbiprofen in Blank FaSSIF, FaSSIF, and SIF-FaSSIF, while similar, were lower than

the solubility at pH 6.8 media which has a higher pH value than biorelevant media. Interestingly, neither FaSSIF nor SIF-FaSSIF provided a more significant increase than Blank-FaSSIF. To investigate the food effect on flurbiprofen solubility, we compared the Blank FeSSIF, FeSSIF and SIF-FeSSIF. The solubility of flurbiprofen was increased seven-fold in FeSSIF compared with Blank FaSSIF. However, in SIF-FeSSIF flurbiprofen was eight times more soluble than FeSSIF and 56 times more soluble than Blank-FaSSIF. This dramatic variation between FeSSIF and SIF-FeSSIF is thought to result from the preparation processes. FaSSIF and FeSSIF were prepared using dichloromethane as a solvent and after the emulsification process, the organic solvent should be evaporated as reported in the literature (Dressman et al. 1998). Therefore, it is difficult to standardize the preparation method. It may be affected by the capacity of the equipment in the emulsification and evaporation processes. However, the SIF-Powder was simply dissolved in the blank mediums as described in Biorelevant media. Therefore, probable variations arising from using different analysts, equipment or time can be minimized.

Based on the D_o values given in Table 3, flurbiprofen was found to have low solubility in pH 4.5 and blank FaSSIF (D_0>1), while it was highly soluble (D_0<1) in all the other media (Table 3).

Table 3. Comparing the flurbiprofen dissolution curves in different media

Test media	Reference media	f_2 value*	Similarity
Blank FaSSIF	FaSSIF	64.2	Similar
Blank FaSSIF	SIF-FaSSIF	63.3	Similar
FaSSIF	SIF-FaSSIF	54.0	Similar
Blank FeSSIF	FeSSIF	36.2	Not similar
Blank FeSSIF	SIF-FeSSIF	16.3	Not similar
FeSSIF	SIF-FeSSIF	27.2	Not similar

*When similarity factor, f2≥50 dissolution curves are similar
FaSSIF: Fasted State Simulated Intestinal Fluid, FeSSIF: Fed State Simulated Intestinal Fluid , SIF-FaSSIF: Fasted State Simulated Intestinal Fluid prepared using instant powder, SIF-FeSSIF: Fed State Simulated Intestinal Fluid prepared using instant powder, Blank FaSSIF: Fasted State Simulated Intestinal Fluid media not including the bile salts and phospholipids, Blank FeSSIF: Fed State Simulated Intestinal Fluid media not including the bile salts and phospholipids

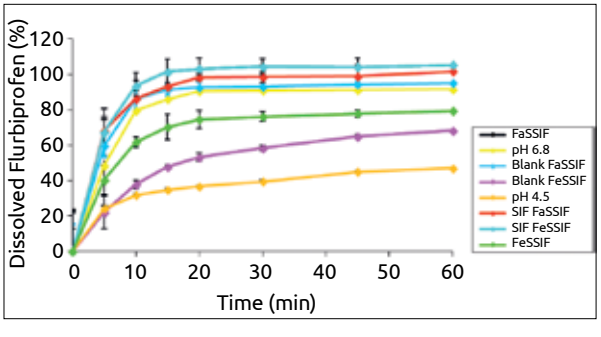

Figure 1. Dissolution profiles of flurbiprofen tablets

In vitro dissolution studies

Dissolution studies were performed to witness the media effects on BCS Class 2 drug. Ansaid® (Pfizer, Turkey) 100 mg was used for the dissolution studies as a reference product for flurbiprofen. The mean dissolution profiles of flurbiprofen are shown in Figure 1. Since flurbiprofen is a weak acid with a pK_a of 4.22, the ionized form of flurbiprofen increases as the pH of the dissolution media increases. After 1 hour, the dissolved ratio of flurbiprofen from tablets was 47% at pH 4.5 buffer, whereas the dissolved amount was 68% in Blank FeSSIF (pH 5.0). Although, the dissolution of flurbiprofen increased with a pH higher than pK_a of 4.22, a complete dissolution was not observed at pH 5.0. Given the use of SIF-FeSSIF containing bile salt/lecithin media with the same pH, a great enhancement resulting in complete dissolution was observed. Nevertheless, in FeSSIF, media which includes the same amount of bile salt and lecithin, a complete dissolution was not achieved. When comparing FeSSIF and SIF-FeSSIF, the two-bile salt and lecithin containing media, this significant difference is thought to result from the preparation conditions. SIF-FeSSIF is an easy-to-prepare instant powder, whereas FeSSIF involves a complicated preparation process including an organic solvent evaporation step. The similarity factors of the biorelevant media and their blanks are given in Table 3. More than 85% of the drug was released in 30 minutes at pH 6.8, Blank FaSSIF, FaSSIF and SIF-FaSSIF with pH 6.5. While the pH effect was predominant at the higher pH values, bile salt effect was dominant at the lower pH levels. Therefore, thanks to the pH of FaSSIF 6.5, it may not be necessary to use the bile salts in media. However, the dissolution results of FeSSIF, Blank FeSSIF and SIF-FeSSIF were not found to be similar. SIF-FeSSIF significantly increased the release of flurbiprofen. This means that the preparation method is equally as important as the composition of the fed state biorelevant media. In line with these results, we decided to assess the preparation method of the biorelevant media when evaluating the cytotoxicity on the Caco-2 cell line.

Cytotoxicity results

The effects of sodium taurocholate and lecithin, DMSO, and flurbiprofen on the mitochondrial dehydrogenase activity were studied in the Caco-2 cell monolayers. Conventionally prepared biorelevant media (Dressman et al. 1998), and the media prepared using SIF-Powder® (the instant powder), were evaluated separately. To eliminate the pH effect, the media without bile salt and phospholipid (Blank media) were also investigated and DMEM without serum was used as a control. Additionally, a model drug (flurbiprofen) at two different concentrations (100 µM and 500 µM) was evaluated in these biorelevant media. Cell viability was scored according to the following classification (Dahl et al. 2006): More than 90 percent of cell viability was defined as "non-cytotoxic"; 60-90 percent of cell viability was defined as "slightly cytotoxic"; 30-59 percent of cell viability was defined as "moderately cytotoxic" and less than 30 percent of cell viability was defined as "severely cytotoxic".

Since the model drug possesses a high permeability, the initial measurement was carried out at the first hour and

another measurement was performed, at the 24th hour. The cell viability percents of cells in Blank FaSSIF, FaSSIF and SIF-FaSSIF were 88.4%±7.2, 93.7%±6.7 and 91.3%±2.7 after 1 hour and 73.2%±1.5, 78.5%±1.6 and 77.5%±8.0 after 24 hours, respectively (Table 4). According to the cytotoxicity classification, it was accepted as "slightly cytotoxic". When Blank FaSSIF without bile salts was compared with FaSSIF and SIF-FaSSIF which included 3 mM sodium taurocholate and 0.75 mM lecithin, no difference was observed. Moreover, the viability results at 24 hours were nearly 15% lower than the results at 1 hour. The viability percents of cells in Blank FeSSIF, FeSSIF and SIF-FeSSIF were 86.1%±2.4, 84.3%±4.2 and 82.4%±4.8 after 1 hour and 70.8%±8.2, 73.2%±3.0 and 69.1%±4.4 after 24 hours, respectively. According to the cytotoxicity classification, they were accepted as "slightly cytotoxic". When Blank FeSSIF without bile salts were compared with FeSSIF and SIF-FeSSIF which included 15 mM sodium taurocholate and 3.75 mM lecithin, no difference was observed. However, the viability results at 24 hours were lower by between 10-15% when compare with the results at 1 hour (Figure 2). This decrease may result from the different pH of DMEM (pH 7.4) and the biorelevant media (pH 6.5 for FaSSIF and pH 5.0 for FeSSIF). Antoine et al. (2015) found similar results with 83%±24 and 69%±17 of cell viability after 2 hours using FaSSIF and FeSSIF, respectively. In another study (Patel et al. 2006), while cell viability results were close to our results for FaSSIF (nearly 70%), FeSSIF was found very cytotoxic on cells with about 10% of viability after 2 h. For this reason, Patel et al. (2006) recommended modifications on FeSSIF when using in permeability studies. Ingels and Augustijns (2003) found 96.9% ± 20.1 cell viability for FaSSIF, but very low cell viability (5.4%±0.3) for FeSSIF. These results are conflicting and none of them were comparable with the blank media. Therefore, our blank-controlled study has given us more reliable results. The cell viability results are given in Table 4. The cytotoxicity results of flurbiprofen in concentrations of 100 µM and 500 µM were also similar to the results of the biorelevant media without the drug. The results were between 80-94% (mean value of 86.3±6.3%) after 1 hour and 72-87% (mean value of 79.6±6.0%) after 24 hours. These results have shown us that no additional toxic effect associated with the drug was obtained considering the viability results of cells (Figure 3).

Table 4. Cell viability of the biorelevant media and their blanks

	Cell viability (%) after 1 h	Cell viability (%) after 24 h
Blank FaSSIF	88.4±7.2	73.2±1.5
FaSSIF	93.7±6.7	78.5±1.6
SIF-FaSSIF	91.3±2.7	77.5±8.0
Blank FeSSIF	86.1±2.4	70.8±8.2
FeSSIF	84.3±4.2	73.2±3.0
SIF-FeSSIF	82.4±4.8	69.1±4.4

FaSSIF: Fasted State Simulated Intestinal Fluid, FeSSIF: Fed State Simulated Intestinal Fluid , SIF-FaSSIF: Fasted State Simulated Intestinal Fluid prepared using instant powder, SIF-FeSSIF: Fed State Simulated Intestinal Fluid prepared using instant powder, Blank FaSSIF: Fasted State Simulated Intestinal Fluid media not including the bile salts and phospholipids, Blank FeSSIF: Fed State Simulated Intestinal Fluid media not including the bile salts and phospholipids

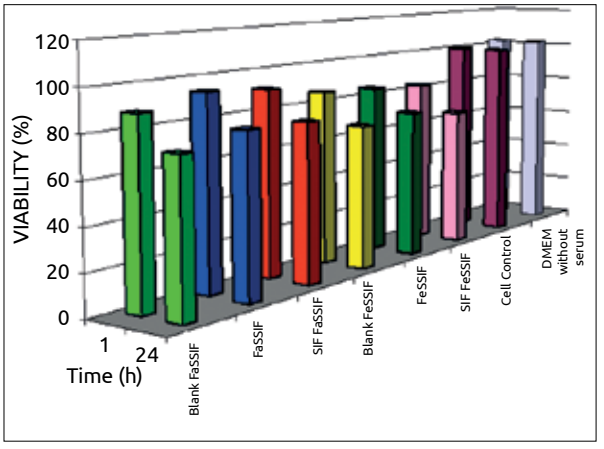

Figure 2. Viability of different dissolution media

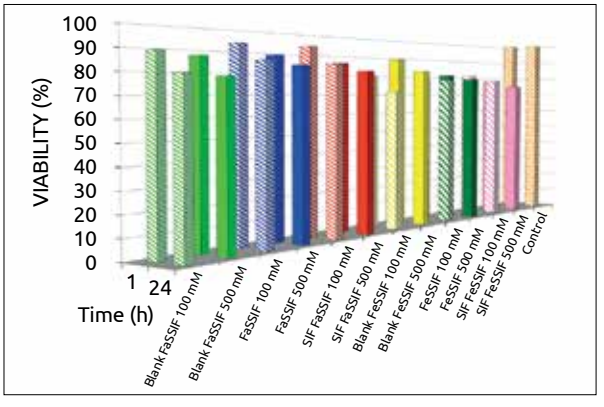

Figure 3. Viability of flurbiprofen in different dissolution media

As flurbiprofen is a weak acid, its solubility was affected by both pH and the bile salt content of the dissolution media. The effect of the bile salt content was found more significant in the media at pH values lower than the pKa of flurbiprofen, while the pH effect was more distinctive at higher pH values. Likewise, biorelevant media which was prepared using SIF-Powder®, gave more notable results in solubility and dissolution studies of flurbiprofen. Moreover, the decision to apply a biowaiver can be considered for flurbiprofen which is a weak acid and may be completely absorbed in small intestine due to its high solubility, high permeability and rapid dissolution properties in pH 6.8 and biorelevant simulated intestinal fluids. On this point, using the biowaiver media in tests for BCS 2 drugs would be important to enhance the solubility and dissolution rate. As for the results of MTT tests on Caco-2 cells, there was no significant difference between the biorelevant media prepared conventionally and media prepared using the instant powder. Additionally, no meaningful difference was observed between the biorelevant media and their blanks in the Caco-2 cell viability. This means that, contrary to expectations, the main effect on cell viability was pH, with regard to bile salt and lecithin effects. Although, the cell

viability results for the media which reflect the fasted state were higher than the fed state media, all media were categorized as "slightly cytotoxic" with about 70-90% of cell viability. Therefore, all media might be used in Caco-2 permeability studies. The viability results of cells decreased in the ratio of 10-15% after 24 hours compared to the results at 1 hour. DMEM, a routinely used media, has a pH of 7.4 whereas the studied pH values for fasting and fed states were 6.5 and 5.0, respectively. This decrease in the viability results was related to the lower pH values. The cytotoxicity results using a model drug -flurbiprofen (BCS Class 2) - in two different concentrations were similar to the viability results of the media without any drug. This finding suggests that model drugs had no effect on cell viability.

In conclusion, biorelevant media is a solid method of approach in evaluating in vitro drug performance and development of new drug pharmaceuticals. However, the preparation of the method of the media is also vital for determining the efficiency of the tests. In that respect, dissolution media prepared from instant powder (such as SIF Powder®) are more useful for low soluble drugs. We also saw a dramatic difference in dissolution and solubility results in the fed state of flurbiprofen - a Class 2 drug. Furthermore, these media can be used for safely performing permeability studies since the bile salts and lecithin did not cause any cytotoxic effect on the Caco 2 cells. However, minor changes in pH of these media can affect the results. Therefore, pH adjustment should be considered in further investigations.

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Original Article

Biological, phytochemical, and physico-chemical properties of two commercial *Nigella sativa* seed oils: A comparative analysis

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ABSTRACT

This study was designed to evaluate and correlate the biological, phytochemical, and physicochemical properties of two NSS (*Nigella sativa* seed) oils (N1 and N2). The biological activity was evaluated in terms of antibacterial, antioxidant (nitric oxide scavenging), antielastase, antityrosinase, antimelanogenic, and anticancer activity. Physicochemical properties including colour, total soluble solids (TSS), and density were also investigated. N2 exhibited higher antibacterial, NO scavenging, extracellular antimelanogenic, and anticancer activity against the HeLa cell line compared to N1, which showed higher antityrosinase activity. TPC (total phenolic content) and TFC (total flavonoid content) of N1 were significantly higher than N2 while TTC (total tannin content) was higher in N2. In fact, TTC was strongly correlated (R=1.000) with the antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity. To conclude, NSS oil may be considered as a complementary and alternative therapy in the management of infectious and chronic diseases but warrants further *in vivo* pharmacological validation and toxicological analysis.

Keywords: *Nigella sativa*, biological, phytochemical, physicochemical

INTRODUCTION

The growing number of bacterial infections and antibiotic resistance have become a major threat to global health, food security, and development. Without effective antibiotics, modern medical treatments including organ transplantations, chemotherapy, and surgeries become much more risky (WHO 2017a). On the other hand, noncommunicable diseases (NCDs) are now the leading cause of death globally, responsible for 40 million deaths each year, equivalent to 70% of all deaths across the world (WHO 2017b). Many NCDs are associated with an increased oxidative stress which is caused by an imbalance between excess free-radical production and endogenous antioxidant levels in the body (Pham-Huy et al 2008). Among NCDs, cancer is the second leading cause of death, accounting for 8.8 million deaths in 2015 (WHO 2017c).

Besides the exploration of antioxidants and chemotherapeutic drugs in the management of NCDs such as cancer, research on the inhibition of key enzymes in the body for the treatment of NCDs has recently intensified. For instance, inhibition of tyrosinase, a key enzyme involved in melanin biosynthesis, may prevent excess formation and accumulation of melanin in the skin, preventing hyperpigmentation disorders including melasma, freckles, lentigines, and geriatric pigment spots (Ya

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et al 2015; Aumeeruddy et al 2017). In addition, over-enzymatic activity, particularly elastase, is associated with loss of skin elasticity; one of the classical aging characteristics. Elastase breaks down elastin, a constituent of the connective tissue responsible for skin firmness and elasticity (Mathen et al 2014).

Recently, much attention has been focused on the exploration of natural remedies for therapeutic purposes due to their low cost, and because of the association of side effects to synthetic drugs. Among natural resources such as terrestrial and marine plants, animals, and micro-organisms, plants have always been a versatile source of shelter, clothing, food, flavours and fragrances, and not the least, medicines (Gurib-Fakim 2006). In fact, 11% of the 252 drugs, which are considered as basic and essential by the World Health Organisation, are exclusively of plant origin or synthetic drugs derived from natural precursors (Rates 2001). In addition, phytochemistry has become a field of active interest for drug discovery and formulations through isolation, purification and characterisation of new phytochemicals, the biologically active compounds found in different parts of plants (Ahmad et al 2013).

Nigella sativa seeds (NSS), also known as black seed (English), çörek otu (Turkish) habat-ul-sauda (Arabic) and kalonji in South Asia, is the black coloured, funnel shaped seeds of the *N. sativa* plant which belongs to the Ranunculaceae family. The plant is cultivated in various regions such as Southern Europe, North Africa, Middle Eastern Mediterranean and the southern regions of Asia including Syria, Turkey, India, Pakistan, and Saudi Arabia (Gilani et al 2004). NSS is regarded as a valuable traditional remedy and has been found to possess extensive biological properties, including antimicrobial, antioxidant, antiinflammatory, anticancer, antidiabetic, cardioprotective properties amongst others (Ahmad et al., 2013). Nonetheless, due to variations observed among studies because of geographical origin, climatic conditions, variety, agricultural techniques applied, extraction and processing techniques, and storage conditions, further analysis is required to understand the factors responsible for these variations in order to obtain better medicinal products and enhanced therapeutic efficacy. The present study therefore aimed to determine any differences in biological activity of two NSS oils originating from the same country, and correlate the data obtained in relation to their phytochemical composition and physicochemical properties. Biological activity was assessed in terms of antibacterial, antioxidant, antielastase, antityrosinase, antimelanogenic, and anticancer activity.

MATERIALS AND METHODS

Materials

Commercially available cold-pressed (expelled) NSS oil (N1) and NSS oil (N2), unspecified regarding its extraction technique, were obtained from shops in Mauritius. The NSS oil samples were stored at room temperature in the dark for the entire duration of the study.

Reagents

All chemicals and reagents used in the study were of analytical grade and were purchased from reliable firms and institutes. Porcine pancreatic elastase type IV, N-succinyl-(Ala)3-p-nitroanilide, Trizma base, XTT cell proliferation kit II, L-ascorbic acid, and Actinomycin D were obtained from Sigma Aldrich, MO, USA. Mushroom tyrosinase, L-tyrosine and koijc acid were obtained from Sigma Aldrich, Johannesburg, RSA. The human cervical adenocarcinoma (HeLa) and human breast adenocarcinoma (MCF-7) cell lines were obtained from the European Collection of Cell Cultures (ECACC, England, UK). Minimum Essential Medium (MEM), trypsin-EDTA, fetal bovine serum (FBS), phosphate buffer saline (PBS), Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), and antibiotics were supplied by Thermofisher scientific (Modderfontein, Johannesburg, RSA). Sodium nitroprusside and Griess-Ilosvsy's nitrite reagent were purchased from Merck Millipore, Darmstadt, Germany.

Antibacterial assays

Disc and well diffusion methods were performed following the guidelines of "The Clinical and Laboratory Standards Institute (CLSI)" (CLSI 2015). The two assays were carried out in parallel and parameters including inoculum level, depth of agar, and size of disc and well, were kept constant. Measurements were carried out in triplicate.

Microorganisms used for antibacterial assay

Clinical isolates including *Proteus* sp., *Klebsiella* sp., *Streptococcus* sp., *Pseudomonas* sp., and *Escherichia coli* were obtained from the Faculty of Science, University of Mauritius, while American Type Culture Collection (ATCC) strains including *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* ATCC 35984, and *Staphylococcus epidermidis* ATCC 14990 were obtained from the Faculty of Agriculture, University of Mauritius. All strains were sub-cultured on Mueller-Hinton Agar (MHA) and grown in Mueller-Hinton broth (MHB) at 37 °C prior to the day of use.

Disc Diffusion Assay

One hundred microlitres of bacterial culture, adjusted to 0.5 McFarland standard turbidity scale in MHB, was spread evenly on the surface of MHA plates. Paper discs (5 mm), prepared from Whatmann filter paper, were impregnated with 30 µl of NSS oil (undiluted), and placed on the inoculated plates. Discs impregnated with 30 µl of streptomycin, cloxacillin, ampicillin, and chloramphenicol, at concentration 1 mg/ml, were used as positive controls, while sterile distilled water was used as the negative control. Plates were incubated at 37 °C for 24 h and the diameter of zone of inhibition (ZOI) including that of the discs were measured in mm.

Well Diffusion Assay

One hundred microlitres of bacterial culture, adjusted to 0.5 McFarland standard turbidity scale in sterile MHB, was spread

evenly on the surface of MHA plates. Five millimeter diameter wells, sufficiently spaced to avoid overlapping of results, were punched into the surface of the agar using a sterile cork borer followed by addition of 30 µl of NSS oil (undiluted) to each well. Four antibiotics were used as positive control including streptomycin, cloxacillin, ampicillin, and chloramphenicol, at a concentration of 1 mg/mL, while sterile distilled water was used as the negative control. Plates were incubated at 37°C for 24 h and the diameter of the ZOI including that of the well were measured in mm.

Antioxidant assay

Nitric oxide scavenging assay

The nitric oxide (NO) scavenging activity of the samples was measured according to the method described by Mayur et al (2010). The oil samples were prepared by dissolving the oil in ethanol to a starting concentration of 10 mg/mL. To the top row of a 96-well plate, 20 µL of distilled water and 80 µl of the NSS oil sample were added. The NSS oil samples were double diluted to a final concentration ranging from 2000 µg/mL to 15.6 µg/mL. Ten millimolar sodium nitroprusside (50 µL) was added to all the wells followed by incubation at room temperature under light for 90 min. After incubation, Griess-Ilosvsy's nitrite reagent (100 µL) was added to the test wells and distilled water to the colour control wells. The nitrite content was measured at 546 nm after 5 min incubation in the dark. L-ascorbic acid (10 mg/mL) and ethanol was used as the positive and negative controls, respectively. The radical scavenging activity was determined as percentage NO radical-scavenging activity which was calculated by the equation: NO radical-scavenging=[(AC-AS)/AC]×100; where AC is the absorbance of the control solution that contains only NO, and AS is the absorbance of NSS oil samples in NO solution. From these results, the fifty percent inhibitory concentration (IC_{50}) was determined using the GraphPad Prism 4.0 program (GraphPad Software, Inc., CA, USA).

Elastase inhibitory activity

The ability of the NSS oil samples to inhibit porcine pancreatic elastase (PPE) was determined by measuring the release of *p*-nitroaniline from N-succinyl-(Ala)3-p-nitroanilide spectrophotometrically according to the method of Bieth et al (1974) with slight modifications. The reaction mixture contained 100 mM Tris buffer (pH 8.0), 0.5 M HCl, and the test sample (NSS oil and the positive drug control, ursolic acid) which were serially diluted to yield a concentration range of 250-3.13 µg/ml. PPE (5 mM) was then added and the reaction mixture was incubated for 15 min followed by the addition of N-succinyl-(Ala)3-p-nitroanilide (4 mM). A vehicle control where the sample was replaced by methanol was included as the 100% rate, and 0% where the enzyme and substrate were replaced, respectively, by buffer solution. The change in the absorbance of the reaction mixture was measured kinetically at 405 nm for 15 min using KC Junior software and a BIO-TEK Power-Wave XS multiwell plate reader. One unit of elastolytic activity is defined as the release of 1 µM of p-nitroaniline/min. The concentration of NSS oil at which fifty percent of the enzyme was inhibited (IC_{50}) was then calculated.

Tyrosinase inhibitory activity

The antityrosinase assay was performed according to the method described by Mapunya et al (2011), with few modifications. The NSS oil samples were dissolved in 100 µl DMSO to a 20 mg/ml stock solution which was diluted with 50 mM potassium phosphate buffer (pH 6.5). In a 96-well microtitre plate placed on ice, 30 µl of tyrosinase enzyme (333 units/ml in phosphate buffer pH 6.5) was added to 70 µl of varying concentrations of NSS oil, in triplicate. After 5 min of incubation on ice, 110 µl of substrate (2 mM L-tyrosine) was added to all the wells. The final concentrations of the sample and positive control (kojic acid) ranged from 1000 to 1.5 µg/ml. The optical density (OD) was then measured over a period of 30 min at a wavelength of 492 nm using BIO-TEK power Wave XS multi-well plate reader (KC Junior). The fifty percent inhibitory concentration (IC_{50}) was then determined by analysing the resulting data using the software GraphPad Prism 4.0 (GraphPad Softwar, Inc., CA, USA).

Melanin inhibitory activity

B16F10 Melanoma Cell culture

Mouse melanocytes (B16F10) were cultured in complete Minimum Essential Eagle's Medium (MEM), containing 10% FBS, 1.5 g/L $NaHCO_3$, 2 mM L-glutamate, 10 mg/ml streptomycin, and 0.25 mg/ml fungizone.

Measurement of melanin production in Cultured B16F10 Melanoma Cells

The inhibitory effect of NSS oil on melanin production was determined following the Hill method previously described by Matsuda et al (2005). The cultured B16F10 mouse melanoma cells were trypsinised (0.25% trypsin and 0.1% EDTA at 37 °C for 5-10 min) and plated into 24-well plates (5×10^4 cells/well in 1.5 ml of MEM). The plated cells were incubated for 24 h at 37 °C in the CO_2 incubator. Following incubation, 500 µl of each NSS oil sample (concentration ranging from 500 to 15.6 µg/ml) was added to each well in duplicate, and the treated 24-well plates were incubated for 3 days at 37 °C in the CO_2 incubator. Test samples and theophylline (negative control) were dissolved in DMSO. The final concentration of DMSO was 5%. The untreated cells were used as the control group.

After incubation, the cultured medium was removed by a pipette and assayed for extracellular melanin as follows: The cultured medium was centrifuged (900 g, 20 min at 4 °C) to separate the cellular components and extracellular components. One millilitre of a mixture of 0.4 M Tris buffer (pH 6.8) and ethanol (9:1, v/v) was added to 1 ml of the supernatant. The OD of the resulting solution was then measured at 475

nm, and the amount of extracellular melanin was determined.

To determine the intracellular melanin production, the remaining melanoma cells were washed with CMF-D-PBS (Calcium and Magnesium Free Dulbecco's-Phosphate Buffered Saline) and trypsinised (100 µl of 0.25% trypsin and 0.1% EDTA at 37 °C for 5-10 min). The cells were digested by the addition of 400 µl of 1 N NaOH and then left standing for 16 h at room temperature. The OD of the resulting solution was then measured at 475 nm, and the amount of intracellular melanin was determined. Melanin inhibition was determined by comparing the OD of the dose dependent treated cells with the untreated cells and the IC_{50} values were determined.

Anticancer activity

Cell culture

The human breast adenocarcinoma (MCF-7) and human cervical adenocarcinoma (HeLa) cell lines were maintained in MEM supplemented with 10% FBS, 1% antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), and 250 µg/ml fungizone. The cells were grown statically at 37 °C in a humidified incubator set at 5 % CO_2. Once confluent, the cells were sub-cultured by treating them with trypsin-EDTA (0.25 % trypsin containing 0.53 mM EDTA) for a maximum of 15 min.

MCF-7 and HeLa cell inhibition

The cytotoxicity of NSS oil was evaluated using the XTT cell proliferation Kit II according to the method of Zheng et al (2001). MCF-7 and HeLa cells (1 x 10^5 cells/ml) were seeded in 96-well microtiter plates, respectively, and allowed to attach for 24 h at 37 °C and 5 % CO_2. The NSS oil samples were prepared at 20 mg/ml stock concentrations in DMSO. The cells were treated with NSS oil at concentrations ranging from 400-3.13 µg/ml and the positive drug control, actinomycin D, with concentrations ranging between 0.5 µg/ml and 0.002 µg/ml. A vehicle control was included where cells were treated with 2 % DMSO. The treated cells were incubated for 72 h followed by the addition of 50 µl XTT to a final concentration of 0.3 mg/ml. The plates were incubated with the viability reagent for 2 h and the absorbance of the colour complex was measured at 490 nm with a reference wavelength set at 690 nm for XTT using KC Junior software and a BIO-TEK Power-Wave XS multi-well plate reader. The assay was performed in triplicate and the fifty percent inhibitory concentration (IC_{50}) values of the samples were calculated using the GraphPad Prism 4.0 program (GraphPad Software, Inc., CA, USA).

Phytochemical analysis

Qualitative phytochemical test

Phytochemical screening for the presence of alkaloids, flavonoids, saponins, phenols, anthraquinones, and steroids was performed using standard protocols (Andzouana and Mombouli 2011; Tiwari et al 2011).

Quantitative phytochemical test

Total phenolic content (TPC) was measured using the Folin–Ciocalteu method as described by Picot et al (2014). Five hundred microlitres of test sample was mixed with 2500 µl Folin–Ciocalteu reagent (ten-fold diluted) and 2000 µl of sodium carbonate solution (7.5 %). The mixture was allowed to stand for 30 min and the absorbance of the solution was measured spectrophotometrically at 760 nm. All determinations were performed in triplicate. TPC was expressed as microgram of gallic acid equivalent (GAE) per gram of sample (µg GAE/g sample).

The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method previously described by Picot et al (2014) . The reaction mixture containing 2 ml of diluted sample and 2 ml of 2 % $AlCl_3$ solution was allowed to stand at room temperature for 30 min after which the absorbance of the solution was measured spectrophotometrically at 420 nm. All determinations were performed in triplicate and TFC was expressed as µg of rutin equivalent (RE) per g of sample (µg RE/g sample).

Total tannin content (TTC) was determined using the vanillin-HCl method as previously described by Mak et al (2013). 1 ml of sample was mixed with 5 ml of the reagent mixture (4 % vanillin in methanol and 8 % HCl in methanol in the ratio of 1:1). After 20 min, the resulting color change was measured spectrophotometrically at 500 nm. TTC was expressed as µg catechin equivalent (CE) per g sample (µg CE/g sample).

Physicochemical properties

Physicochemical properties including colour, TSS, and density were tested. For colour measurement, CIELAB L* a* b* colour parameters were determined using a chromameter (Minolta CR-410, Konica Minolta, Japan), which was placed directly over the samples in petri dishes filled to the brim. L* represents lightness, a* measures the degree of red (+a*) or green (-a*) colours and b* parameter indicates the degree of the yellow (+b*) or blue (-b*) colours (Boussaid et al 2014). In addition, TSS was measured using a digital hand-held "Pocket" refractometer (ATAGO, PAL-3) with ranges of 0-93˚Brix. Density was measured according to the method described by Kinoo et al (2012) using the formula: Density = Mass of sample/volume of sample. All measurements were done in triplicate.

Statistical analysis

All data presented in this study was analysed using Microsoft Excel 2010, Minitab version 16, and GraphPad Prism 4.0. One way ANOVA (Tukey's test) was used for evaluation of significant differences between the variables. Pearson's correlation was used to evaluate correlation between the variables. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Antibacterial activity

The results of the antibacterial activity of NSS oil are shown in Table 1 and Table 2. The bacterial strains displayed variation

Table 1. Antibacterial activity of undiluted samples using disc diffusion assay

	N1	N2	Streptomycin	Ampicillin	Cloxacillin	Chloramphenicol
E.coli (clinical isolate)	7.3±0.58^{c}	8.0±0.0^{c}	10.7±0.58^{b}	NI	NI	21.0±1.0^{a}
E.coli ATCC 25922	10.0±1.0^{c}	10.3±0.58^{c}	25.3±0.58^{a}	13.0±0.0^{b}	NI	25.7±0.58^{a}
Proteus sp. (clinical isolate)	8.7±0.58^{c}	9.0±0.0^{c}	18.7±0.58^{a}	NI	NI	15.7±0.58^{b}
P. mirabilis ATCC 12453	33.0 ±1.0^{b}	≥ 44^{a}	23.7±0.58^{e}	20.0±1.0^{f}	28.7±0.58^{c}	26.7±0.58^{d}
Pseudomonas sp. (clinical isolate)	7.7 ±0.58^{a}	8.0±1.0^{a}	NI	NI	NI	NI
P. aeruginosa ATCC 27853	15.3±0.58^{d}	31±1.0^{a}	25.7±0.58^{b}	15.3±0.58^{d}	13.7±0.58^{e}	23.0±0.0^{c}
Klebsiella sp. (clinical isolate)	8.0±1.0^{b}	8.7 ± 0.58^{b}	16.3±0.58^{a}	NI	NI	8.0±0.0^{b}
Streptococcus sp. (clinical isolate)	19.7±0.58^{b}	39.7 ± 0.58^{a}	NI	16.0±1.0^{c}	NI	21.0±1.0^{b}
S. epidermidis ATCC 35984	39.7±0.58^{b}	≥ 44^{a}	NI	12.7±0.58^{d}	9.0±1.0^{e}	24.3±0.58^{c}
S. epidermidis ATCC 14990	29.7±0.58^{c}	≥ 44^{a}	20.7±0.58^{e}	19.7±0.58^{e}	38.7±0.58^{b}	23.7±0.58^{d}

Diameter of inhibition zones includes diameter of discs (5 mm); Values represent mean of triplicate±standard deviation (n=3) in mm; NI: No inhibition; Values ≥ 44 indicates measurement of radius x 2 because of too large and overlapping ZOI; N1: NSS oil sample 1; N2: NSS oil sample 2; All antibiotics were tested at 1 mg/mL; Different letter superscript between columns means significantly different ($p<0.05$).

Table 2. Antibacterial activity of undiluted samples using well diffusion assay

	N1	N2	Streptomycin	Ampicillin	Cloxacillin	Chloramphenicol
E.coli (clinical isolate)	NI	NI	12.3±0.58^{b}	NI	NI	18.3±0.58^{a}
E.coli ATCC 25922	NI	NI	28.3±0.58^{a}	16.3±0.58^{b}	NI	29.7±0.58^{a}
Proteus sp. (clinical isolate)	9.3±0.58^{d}	11.0±0.0^{c}	21.7±0.58^{a}	NI	NI	18.3±0.58^{b}
P. mirabilis ATCC 12453	23.0±1.0^{e}	39.7±0.58^{a}	28.3±0.58^{d}	29.3±0.58^{cd}	30.0±0.0^{bc}	31.3±0.58^{b}
Pseudomonas sp. (clinical isolate)	NI	NI	NI	NI	NI	NI
P. aeruginosa ATCC 27853	13.3±0.58^{d}	23.7±0.58^{b}	26.3±0.58^{a}	17.7±0.58^{c}	16.7±0.58^{c}	24.7±0.58^{b}
Klebsiella sp. (clinical isolate)	NI	NI	17.0±0.0^{a}	NI	NI	9.0±0.0^{b}
Streptococcus sp. (clinical isolate)	11.7±0.58^{c}	23.0±1.0^{ab}	NI	22.3±0.58^{b}	NI	24.7±0.58^{a}
S. epidermidis ATCC 35984	38.3 ± 0.58^{b}	≥ 44a	NI	13.7±0.58^{d}	13.3±0.58^{d}	30.3±0.58^{c}
S. epidermidis ATCC 14990	21.7±0.58^{e}	33.7±0.58^{b}	23.3±0.58^{d}	20.3±0.58^{e}	41.3±0.58^{a}	27.0±1.0^{c}

Diameter of inhibition zones includes diameter of discs (5 mm); Values represent mean of triplicate±standard deviation (n=3) in mm; NI: No inhibition; Values ≥ 44 indicates measurement of radius x 2 because of too large and overlapping ZOI; N1: NSS oil sample 1; N2: NSS oil sample 2; All antibiotics were tested at 1 mg/mL; Different letter superscript between columns means significantly different ($p<0.05$).

Table 3. Other biological activities of NSS oil

Samples	NO scavenging	Elastase inhibition	Tyrosinase inhibition	Melanin inhibition		Anticancer	
				Intracellular	Extracellular	MCF-7 cell line inhibition	HeLa cell line inhibition
	IC50 (µg/mL)						
N1	176.8±3.2^{a}	NIA	544.6±1.915^{b}	NIC	NIC	NID	NID
N2	148.1±5.8^{b}	NIA	NIB	NIC	47.83±1.680^{a}	NID	375.2± 9.9^{b}
L-Ascorbic acid	66.4±1.9^{c}	NT	NT	NT	NT	NT	NT
Ursolic acid	NT	4.27±0.65	NT	NT	NT	NT	NT
Kojic acid	NT	NT	2.849±4.469^{a}	NT	NT	NT	NT
Arbutin	NT	NT	NT	99.57±1.998	99.57±1.998^{b}		
Actinomycin D	NT	NT	NT	NT	NT	0.0075±3.9	0.0022±3.4^{a}

Values represent mean of triplicate ± standard deviation (n=3) in µg/mL, N1: NSS oil sample 1; N2: NSS oil sample 2; Different letter superscript (lowercase) within column means significantly different ($p<0.05$). IC50: Fifty percent inhibitory concentration,

A: No inhibition at the highest concentration tested of 250 µg/mL

B: No inhibition at the highest concentration tested of 1000 µg/mL

C: No inhibition at the highest concentration tested of 500 µg/mL

D: No inhibition at the highest concentration tested of 400 µg/mL

NI: No inhibition

NT: Not tested

in susceptibility to the tested samples. In general, the ATCC strains were found to be more susceptible compared to the clinical isolates. Overall, N2 was more effective than N1, exerting significantly ($p<0.05$) greater antibacterial activity against *P. mirabilis* ATCC 12453, *P. aeruginosa* ATCC 27853, *Streptococcus* sp. and *S. epidermidis* (ATCC 35984 and ATCC 14990) compared to the reference antibiotics tested, which were found to be ineffective against several tested bacteria (Table 1 and Table 2). For instance, 6 out of 10 bacterial strains were resistant to cloxacillin while 4 strains were resistant to ampicillin. On top of that, the *Pseudomonas* sp. (clinical isolate) was resistant to all 4 antibiotics. The effectiveness of NSS oil against the tested bacteria in the present study is in conformity with the findings of previous studies (Arici et al 2005; Salman et al 2008). However, in contrast to the sensitivity of *Klebsiella* sp. to NSS as observed in our study, the study of Salman et al (2008) showed that NSS oil was not effective against ten multi-drug resistant strains of *K. pneumoniae*. Other *Klebsiella* sp. such as *K. planticola* were also found to be resistant to the oil (Shafi et al 2009). Additionally, resistance to *E. coli* was observed by Salman et al (2008) in contrast to the findings of the current study. This variation in activity can be due to several factors including geographical regions, plant growing conditions, extraction techniques, processing, refining, and purification of the crude oil which may destroy the antibacterial compounds of the oil.

Comparison of the two antibacterial assays revealed significant differences such that the NSS oil samples displayed significantly greater ZOI ($p<0.05$) against all tested bacteria using the disc diffusion method except *Proteus* sp. (clinical isolate) for which the well diffusion assay was found to be better (Fig. 1). For instance, no ZOI was observed in well diffusion assay against the two tested *E.coli* strains, *Pseudomonas* sp. and *Klebsiella* sp. while a small ZOI in the range 7.3-10.3 mm was observed using disc diffusion method. The higher activity of the oil in disc diffusion compared to the well diffusion assay in our study could be due to the direct contact of the disc (impregnated with oil) with the bacteria, indicating a shorter distance for diffusion of the components of the oil through the medium. In contrast, in the well diffusion assay, the bioactive compounds need to diffuse through a longer distance from the bottom of the well to reach the bacteria on the surface of the agar.

Antioxidant activity

The antioxidant activity of the tested NSS oil in terms of their scavenging activity against NO radical are shown in Table 3. Among the tested samples, N2 (IC_{50}=148.1 µg/ml) displayed significantly greater scavenging activity than N1 (IC_{50}=176.8 µg/ml) ($p<0.05$). However, the two NSS oil were found to exhibit significantly lower scavenging activity compared to the positive control, L-Ascorbic acid (IC_{50}=66.4 µg/ml) ($p<0.05$). A previous study by Zaher et al (2008) observed a lower IC_{50} value of 110 µg/ml for NSS aqueous extract, hence greater scavenging activity compared to the present study. In addition, NSS aqueous extract was found to exhibit an inhibitory effect on NO production by murine macrophages (Mahmood et al 2003). However, it should be highlighted that few studies have investigated the NO radical scavenging activity of NSS compared to other antioxidant assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging (Goga et al 2012; Haron et al 2014; Feroz and Uddin 2016), indicating the necessity of conducting more antioxidant assays to obtain the complete antioxidant profile of NSS.

Elastase inhibitory activity

The inhibitory effect of NSS oils on elastase activity are displayed in Table 3. At the highest concentration tested (250 µg/ml), no inhibition was observed in contrast to the positive control, ursolic acid, which displayed an IC_{50} value of 4.27 µg/ml. On the contrary, Kacem and Meraihi (2006) found that the essential oil of Algerian NSS showed dose-dependent inhibition on human neutrophil elastase. An increase in activity was observed from 410 µg/ml (about 20% inhibition) to 5800 µg/ml (total inhibition). Therefore, a concentration higher than that used in the present study might show potential antielastase activity which needs to be confirmed by future studies.

Tyrosinase inhibitory activity

The tyrosinase inhibitory activity of the two NSS oil samples are presented in Table 3. At the highest concentration tested, 1000 µg/ml, N2 did not show any inhibitory activity. On the other hand, N1 inhibited 50% of the enzyme at a concentration of 544.6 µg/ml, but was less effective compared to the positive control kojic acid (IC_{50}=2.849 µg/ml). Few studies have demonstrated the antityrosinase activity of NSS. For instance, an inhibition of 49.6 % was observed by the chloroform: methanol (2:1) extract of NSS at 500 µg/ml (Subramanian and Sahithya 2016). In addition, Muddathir et al (2017) found that the methanolic NSS extract (500 µg/ml) exhibited tyrosinase inhibition of 15.73 % and 17.64 % to L-tyrosine and L-DOPA, respectively, while no inhibition was observed at 125 µg/ml. Interestingly, the study of Sekeroglu et al (2012) revealed a lower active concentration of 50 µg/ml, showing a dose-dependent tyrosinase inhibitory activity.

Melanin inhibitory activity

The IC_{50} values obtained in the melanin inhibition assay are shown in Table 3. The NSS oil samples displayed no inhibition on intracellular melanin synthesis at the highest tested concentration (500 µg/ml). In contrast, although N1 was ineffective against extracellular melanogenesis at the highest tested concentration (500 µg/ml), a 50% inhibition was observed by sample N2 at a concentration of 47.83 µg/ml, which was more effective compared to the positive control arbutin (IC_{50}=99.57 µg/ml). Compared to previous studies, the antimelanogenic activity of NSS oil observed in the present study was found to be in agreement with that of Lee et al (2011) who found that NSS oil caused a reduction in melanin production up to 86 % at a concentration of 10

mg/ml. Although sample N1 showed no antimelanogenic activity, it should be noted that its tyrosinase activity observed in the present study indicates its indirect role as an antimelanogenic agent due the involvement of the tyrosinase enzyme in melanogenesis (Aumeeruddy et al 2017). It is also important to note that NSS is used as an ingredient in various cosmetic skin products including soap, cream, shampoo, and has been found to possess cosmeceutical properties including anti-hair loss, anti-psoriatic, anti-acne vulgaris, anti-vitiligo, and wound healing properties (Sudhir et al 2016; Eid et al 2017).

Anticancer activity

As shown in Table 3, although NSS oil N1 displayed no anticancer activity on HeLa cells at the highest concentration tested (400 µg/ml), N2 was effective with an IC_{50} value of 375.2 µg/ml. Nonetheless, the positive control actinomycin D (IC_{50}=0.0022 µg/ml) showed higher activity compared to N2. The cytotoxicity of NSS against HeLa cells have also been observed by various studies. For instance, the methanolic, n-Hexane, and chloroform extracts of NSS were found to display IC_{50} values of 2.28 µg/ml, 2.20 µg/ml and 0.41 ng/ml, respectively, inducing apoptosis in HeLa cells (Shafi et al 2009). The ethanol extract was also found to inhibit proliferation and colony formation, and induce apoptosis in HeLa cells. The apoptotic induction was exerted through the release of mitochondrial cytochrome c, increase of the Bax/Bcl-2 ratio, activation of caspases (3, 8, and 9) and cleavage of poly (ADP-ribose) polymerase. Also, it modulated the expression levels of cell cycle-related proteins such as c-Myc, hTER, cyclin D1, CDK-4, p53, and p21 (Elkady 2012).

On the other hand, the two NSS oils tested in the present study showed no inhibitory effect on MCF-7 cell line at a highest tested concentration of 400 µg/ml, which was in disagreement with several studies. For instance, the study of Baharetha et al (2013) revealed that among 12 supercritical carbon dioxide (SC-CO_2) extracts, the extract prepared at higher temperature (60 °C) and lower pressure (2500 psi) showed selective antiproliferative activity against MCF-7 cells with an IC_{50} value of 53.34 µg/ml, and caused significant apoptosis in the cell line by activating caspase 3/7 and 8. Another study (Farah 2005) showed that the ethanolic extract of NSS exerted more potency than the aqueous extract against MCF-7 cells in the presence and absence of H_2O_2. In addition, the cytotoxicity of doxorubicin was also enhanced by co-administration with a nanoemulsion of NSS oil (Mahmoud and Torchilin 2013). It is important to highlight that the NO scavenging activity of NSS oil observed in the present study can prevent the reaction of NO with superoxide ($O_2^{\cdot-}$) to form peroxynitrite (ONOO–), a much more powerful oxidant which can be involved in the aetiology of cancer due to its genotoxicity and damages caused to biomolecules (Pacher et al 2007).

Table 4. Qualitative phytochemical compositions of NSS oils

	Samples	
Phytochemicals	N1	N2
Alkaloids	+	+
Phenols	+	+
Flavonoids	+	+
Saponins	+	+
Steroids	+	+
Anthraquinones	+	+

Note: N1: NSS oil sample 1; N2: NSS oil sample 2

Table 5. Quantitative phytochemical composition of NSS oils

Sample	Total phenolic (µg GAE/g)	Total flavonoid (µg RE/g)	Total tannin (µg CE/g)
N1	769.44±5.63[a]	58.56±1.95[a]	754.10±15.01[b]
N2	530.1±12.98[b]	38.71±1.65[b]	1369.61±14.82[a]

Values represent mean of triplicate ± standard deviation (n=3), N1: NSS oil sample 1; N2: NSS oil sample 2; Different letter superscript within columns means significantly different ($p<0.05$). CE, Catechin equivalent; GAE, Gallic acid equivalent; RE, Rutin equivalent

Phytochemical composition

Preliminary phytochemical screening revealed the presence of all tested phytochemicals in NSS oil including alkaloids, phenols, flavonoids, saponins, steroids, and anthraquinones (Table 4). Regarding the quantitative phytochemical composition (Table 5), variations were observed among the tested samples such that the TPC of N1 (769.44 µg GAE/g) was found to be significantly greater compared to N2 (530.1 µg GAE/g) ($p<0.05$). A similar pattern was observed for TFC (N1=58.56 µg RE/g; N2=38.71 µg RE/g). In contrast, N1 was found to contain lower TTC (754.10 µg CE/g) compared to

Table 6. Correlation between TPC, TFC, TTC, colour, and the observed biological activities

Assay	TPC	TFC	TTC	L*	a*	b*
NO scavenging activity	-1.000	-1.000	1.000	-1.000	1.000	1.000
Tyrosinase inhibitory activity	1.000	1.000	-1.000	1.000	-1.000	-1.000
Extracellular antimelanogenic activity	-1.000	-1.000	1.000	-1.000	1.000	1.000
HeLa cell inhibitory activity	-1.000	-1.000	1.000	-1.000	1.000	1.000

TFC, Total flavonoid content; TPC, Total phenolic content. L* represents lightness, a* measures the degree of red (+a*) or green (-a*) colours and b* parameter indicates the degree of the yellow (+b*) or blue (-b*) colours.

N2 (1369.61 µg CE/g). In fact, a strong positive correlation (R=1.000) was observed between TTC and the antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity of NSS oil, while TPC and TFC (R=1.000) were positively correlated with antityrosinase activity (Table 6). Compared with previous studies, the TPC of NSS in the current study was observed to be lower compared to the range 0.96-7.60 mg GAE/g as found by Haron et al (2014) and Lutterodt et al (2010).

These variations could be attributed to the extraction techniques as well as other reasons mentioned previously, resulting in variations in the chemical compositions of the oil. Although the antibacterial, antioxidant, extracellular antimelanogenic, and HeLa cell line inhibitory activity of sample N2 was found to be higher than N1, N2 displayed lower total phenolic and flavonoid content. This might suggest that compounds found in higher concentrations are not necessarily responsible for the total activity. It might be that the different compounds in small concentrations act synergistically or specific phenolic and flavonoid compounds with stronger bioactivities are present in higher concentration.

Table 7. Physicochemical properties of NSS oil

		N1	N2
pH		ND	ND
Color	L*	66.48±0.64^a	60.48±2.18^b
	a*	9.87±0.49^b	13.07±1.30^a
	b*	46.38±0.19^b	50.42±2.47^a
Total soluble solids (˚Brix)		73.5±1.14^a	73.3±0.49^a
Density (g/ml)		0.85±0.01^b	0.89±0.00^a

Values represent mean of triplicate ± standard deviation (n=3), ND: Not detected; N1: NSS oil sample 1; N2: NSS oil sample 2; (-): not detected; Different letter superscript between columns means significantly different ($p<0.05$). L* represents lightness, a* measures the degree of red (+a*) or green (-a*) colours and b* parameter indicates the degree of the yellow (+b*) or blue (-b*) colours.

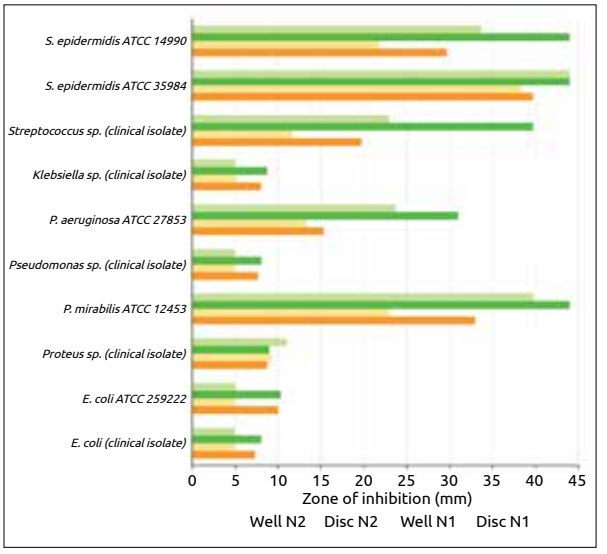

Figure 1. Mean ZOI for disc diffusion and well diffusion assay

Diameter of ZOI includes diameter of disc and well (5 mm); ZOI of 5 mm indicates no inhibition; N1: NSS oil sample 1; N2: NSS oil sample 2

For instance, the higher concentration of tannins observed in N2 compared to N1, might explain the higher antibacterial activity of N2. In fact, tannins isolated from several plants have been found to possess significant antimicrobial activity (Ho et al 2001; Jada et al 2014). Moreover, thymoquinone (TQ) has been found to be a major contributor to the biological activities of NSS including antimicrobial activity (Shohayeb and Halawani 2012). In addition, TQ (about 60% inhibition) and thymohydroquinone (35% inhibition) at 2 µg/ml, were more effective than NSS oil in suppressing melanin production (Lee et al 2011). The mechanisms by which TQ exhibits its anticancer activity against MCF-7 and HeLa cells have also been reported by several studies (Alobaedi et al 2017; Cakir et al 2016; Rajput et al 2013; Woo et al 2011; Yazan et al 2009).

However, it is important to highlight that although TQ has been found to the major contributor to the antioxidant activity of NSS, other constituents, mainly those of the essential oil fraction, including carvacrol, thymol, t-anethole, γ-terpinene, 4-terpineol, p-cymene, β-pinene, and α-thujene, have also been found to be responsible (Ahmad and Beg 2014; Burits and Bucar 2000; Kazemi 2014). Additionally, carvacrol was found to be the most bioactive compound in inhibiting human neutrophil elastase with a very low IC_{50} value (12 µM), followed by carvone (IC_{50}=14 µM), p-Cymene (IC_{50}=25 µM), and TQ (IC_{50}=30 µM) (Kacem and Meraihi 2006). In addition, thymohydroquinone was found to display higher tyrosinase inhibitory activity (68.82% inhibition at 120 µg/ml) compared to TQ (8.24% inhibition at 166.67 µg/ml) (Lee et al 2011). Therefore, the total bioactivity of NSS is not likely to be due to the presence of one main compound but rather due to its plethora of phytochemicals. These bioactive compounds may act in combination to produce synergistic or additive effect which need to be confirmed by future studies.

Physicochemical properties

The physicochemical properties of the tested samples including TSS, colour, and density are shown in Table 7. No significant difference was observed in the TSS (˚Brix: N1= 73.5; N2=73.3) of the two NSS oil tested ($p>0.05$). On the other hand, significant difference was observed in the density of the tested samples such that N2 (0.89 g/mL) exhibited a higher density than N1 (0.85 g/mL) ($p<0.05$). The density of NSS oil was found to be close to that reported by the study of Zzaman et al (2014) (0.93-0.98 g/mL).

Comparison of the colour of the tested samples revealed that N2 displayed significantly lower L* value, higher a* and b* values (L*=60.48, a*=13.07, b*=50.42) than N1 (L*=66.48, a*=9.87, b*=46.38) ($p<0.05$). This may suggest the presence of more yellow pigments (e.g. carotenoids) in N2, which may

have contributed to its higher antioxidant activity compared to N1. Compared to other studies, the two NSS oil in the current study displayed higher L* and a* value, and lower b* value than Tunisian and Iranian NSS oil (Cheikh-Rouhou et al 2007). This discrepancy might be due to difference in seed variety or extraction technique employed. In fact, the study of Zzaman et al (2014) showed that screw pressed NSS oil at different temperatures displayed variations in colour. Interestingly, a* and b* values were positively correlated (R=1.000) with the antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity, while L* was positively correlated with the antityrosinase activity (R= 1.000) (Table 6).

CONCLUSION

From the present investigation, it was found that NSS oil possess major bioactive phytochemicals and significant biological activities including antibacterial, antioxidant, antityrosinase, antimelanogenic, and anticancer activity (against HeLa cells). We observed variations between the two NSS oil samples such that N2 displayed higher antibacterial, antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity compared to N1, which showed higher antityrosinase activity. The higher antibacterial activity of N2 when compared to standard antibiotics in the present study highlights its potential role in effectively slowing the evolution of antibiotic resistance in bacteria. We also found that TTC was positively correlated with most observed activities. The two NSS oil samples also displayed significant variation in physicochemical properties including density and colour whereby the a* and b* values were positively correlated with most observed bioactivities. Nonetheless, it has been argued that *in vitro* models inherently bear limitations and fail to replicate the precise cellular and metabolic conditions of an organism. Therefore, it is necessary to validate the observed biological activities together with toxicological analysis *in vivo* and clinically to obtain the therapeutic dose for the treatment and/or management of communicable and noncommunicable diseases.

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