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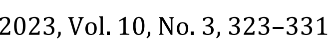

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Synergistic antibacterial evaluation of *Coriandri aetheroleum* and linalool with standard antibiotics

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Abstract: Within this work, it was aimed to investigate the in vitro antibacterial properties of the Pharma grade coriander (*Coriandrum sativum* L.) essential oil, and its combinations. The chemical composition of the essential oil was confirmed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses, simultaneously. Furthermore, the potential antibacterial activity of both the essential oil, and (+)-linalool with standard compounds chloramphenicol, and ciprofloxacin were evaluated using an in vitro microdilution assay against a panel of selected pathogens, namely *Acinetobacter baumannii*, *Bacillus cereus*, *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Initially, the minimum inhibitory concentrations (MIC) were determined, the essential oil as well as (+)-linalool and the standard antibiotics were combined for the synergistic antibacterial activity potential, where the combination activities were expressed as fractional inhibitory concentration index values (Σ FIC). The Coriander oil was relatively more effective against *B. cereus*, *P. aeruginosa*, *E. coli, A. baumannii* (2500 µg/mL) compared to *S. aureus* (5000 µg/mL), respectively. (+)- Linalool was found as effective as the essential oil (2500-5000 µg/mL). Coriander oil and antibiotic combinations showed synergistic effects against *B. cereus* (∑FIC= 0.375), *E. coli* (∑FIC= 0.078) and *S. aureus* (∑FIC= 0.375). Combination with (+)-linalool, and antibiotics showed synergistic effects against *B. cereus* (∑FIC= 0.375), *E. coli* (∑FIC= 0.093), as well as against *S. aureus* (∑FIC= 0.375), respectively. To the best of our knowledge, this is the first time of the antimicrobial combination study of linalool, coriander oil, chloramphenicol, and ciprofloxacin. The initial findings of this work suggest further natural product and drug combination evaluations.

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1. INTRODUCTION

Microorganisms develop resistance to antibiotics due to the unconscious and inappropriate use of resulting in the difficult treatment protocols (Kon & Rai, 2013). Through the intense use of antibiotics, resistant microorganisms emerged such as *Acinetobacter baumannii, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* among other

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pathogens. Diseases caused by multidrug-resistant microorganisms can be treated by combining plant sources with appropriate concentrations of antibiotics, especially with the antimicrobial activity of essential oils (Kon & Rai, 2013). The activity of essential oils can affect both the external envelope of the cell and the cytoplasm. The hydrophobicity of the major antibacterial compositions of essential oils enables partition in the lipids of the cell membranes and mitochondria, disturbing their structures, changing their functions and rendering the permeability (Kon & Rai, 2013).

Coriandrum sativum L. of the Apiaceae is commonly known as a "Coriander" and is originally from Eastern Mediterranean regions, which is cultivated in China, India, Europe, Egypt and Morocco (Ceylan, 1997; Hornok, 1992). It grows naturally in İstanbul, Siirt, Adıyaman, Ankara, Antalya, Çanakkale and Bursa provinces in Turkey (Bakıs *et al*., 2011). This plant is mainly used as spice and flavouring in foods and for the production of its characteristic essential oil. Coriander is known as one of the oldest spice crops in the world (Asgarpanah & Kazemivash, 2012). The plant is used as an antispasmodic, antimicrobial (Kubo *et al*., 2004), antioxidant (Ramadan *et al*., 2003; Bajpai *et al*., 2005) and antidiabetic (Gallagher *et al*., 2003) activities among other biological and pharmacological activities (Hornok, 1992; Asgarpanah & Kazemivash, 2012; Kubo *et al*., 2004; Ramadan *et al*., 2003; Bajpai *et al*., 2005; Gallagher *et al*., 2003). The phytochemical investigations on *C. sativum* have revealed the presence of essential oil, fats and oils, flavonoids, isocoumarins, sterols as well as polyphenolic compounds (Hornok, 1992; Asgarpanah & Kazemivash, 2012; Kubo *et al*., 2004; Ramadan *et al*., 2003; Bajpai *et al*., 2005, Gallagher *et al*., 2003; Özek *et al*., 2010; Beyzi *et al*., 2017). Mostly, the monoterpenoid (+)-linalool (Özek *et al*., 2010) was determined as major component (>50%) in essential oil of Coriander, while α-pinene, camphor, and γ-terpinene are present among others (Asgarpanah & Kazemivash, 2012; Özek *et al*., 2010; Beyzi *et al*., 2017).

In this present study, Pharmacopoeia grade Coriander essential oil (*Coriandri aetheroleum*) acquired from commercial sources was evaluated for its *in vitro* antibacterial properties against food and human pathogenic standard bacterial strains. Not only the oil, but also the main compound linalool, and the combinations with standard antibiotics were subjected for the synergistic potential evaluation, to the best of our knowledge for the first time.

2. MATERIAL and METHODS

2.1. Materials

Pharmacopoeia grade essential oil of Coriander (CO) from Aromapharm Company, Germany, the standard antibiotics chloramphenicol and ciprofloxacin, and (+)-linalool from commercial sources like Sigma-Aldrich (St. Louis, USA) were evaluated for its antibacterial properties, which were in pharmaceutical grade or highest possible purity.

Microorganisms strains (*Acinetobacter baumannii* ATCC 19606, *Bacillus cereus* NRRL B-3711, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC B888, *Staphylococcus aureus* ATCC BAA 1026) used for the evaluation of antibacterial and synergistic activity were obtained from the American Type Culture Collection (ATCC) and Agricultural Research Service Culture Collection (NRRL) in lyophilized form.

2.2. Gas Chromatography (GC) and Gas Chromatography - Mass Spectrometry (GC-MS) analysis

GC and GC-MS conditions were described previously (Demirci *et al*., 2008). Identification of the volatile components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC-MS Library, MassFinder 4.0 Library), (McLafferty & Stauffer, 1989; Koenig *et al*., 2004), and in-house "Başer Library of

Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (Joulain & König, 1998) was used for the identification as also previously reported (Demirci *et al*., 2008).

2.3. Antibacterial Activity

The antibacterial activity of both the essential oil (CO) and the main compound, (+)-linalool (L) (20-0.019 mg/mL) was evaluated by broth microdilution assay according to a modified Clinical and Laboratory Standards Institute (CLSI) method as previously described (CLSI, 2006; Demirci *et al*., 2015). The standard antibiotics chloramphenicol and ciprofloxacin (128-0.25 µg/mL) were used as standard controls. Microbial growth was indicated by change in color from blue to pink with Resazurin. Solvent and microbial controls were also added to the assay plate. Antibacterial assays were repeated at least three times for all the test samples and arithmetic means were reported.

2.4. Synergistic Antibacterial Activity

Interaction of the test samples was studied using the checkerboard microdilution assay in 96-well plates (Van Vuuren *et al*., 2009; Stanojevic *et al*., 2010). Checkerboard method was performed on a 96-well plate using an 8-by-8 well platform. Eight serial dilutions, two-fold dilutions of Coriander essential oil and its main constituent, (+)-linalool (20-0.019 mg/mL) and antibiotics (128-0.25 µg/mL) were prepared. 25 µL aliquots of sample was added to the wells in a vertical orientation, and 25 µL aliquots of each antibiotics dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the two compounds. Positive growth controls (to assess the presence of turbidity) were performed in wells not containing antimicrobial samples. Following this, each well was inoculated with a 50 µL ($5x10^3$ CFU/well) microorganism suspension (turbidometrically standardized), and was further incubated at 35°C for 24 hours. After incubation 20 µL of resazurin was added to all wells and left at 35°C for 2 h, microbial growth was indicated by change in colour from blue to pink. The broth microdilution checkerboard method was performed by using the fractional inhibitory concentration index (∑FIC), which is defined as the sum of the MIC of each sample, when used in combination divided by the MIC of the sample when used alone. Calculations were performed by following equations:

$\sum FIC = FIC\ X + FIC\ Y$

FIC X = (MIC value of combined sample and antibiotic)/(MIC value of antibiotic alone)

FIC Y = (MIC value of combined sample and antibiotic)/(MIC value of sample alone)

Consequently, the activity was defined as follows:

$\sum FIC \leq 0.5$ = synergism;

$\sum FIC\ 0.5 \leq 1$ = additive effect;

$\sum FIC > 1\text{-}4$ = indifferent effect; and as

$\sum FIC \geq 4$ = antagonism for more detailed information check references (Van Vuuren *et al*., 2009; Stanojevic *et al*., 2010) and references herein.

3. RESULTS

3.1. Gas Chromatography (GC) and Gas Chromatography - Mass Spectrometry (GC-MS) Analysis

The essential oil was analyzed to confirm its quality by GC and GC-MS, simultaneously, where the monoterpenes linalool (75 %), *α*-pinene (5 %), camphor (4.5 %), and *γ*-terpinene (3.5%) were characterized as the main constituents, complying with the quality of the supplier and Pharmacopoeia Monograph (2014). Other constituents are given in Table 1.

Table 1. Essential oil constituents of the Coriander oil.

No	RRI[a]	Compound	(%)[b]	IM[c]
1	1032	*α*- Pinene	5.0	tR, MS
2	1076	Camphene	0.8	tR, MS
3	1118	*β*-Pinene	0.4	tR, MS
4	1174	Myrcene	0.5	tR, MS
5	1203	Limonene	2.1	tR, MS
6	1255	*γ*-Terpinene	3.5	tR, MS
7	1280	*p*-Cymene	2.1	tR, MS
8	1290	Terpinolene	0.4	tR, MS
9	1450	*trans*-Linalool oxide	0.1	MS
10	1478	*cis*-Linalool oxide	0.1	MS
11	1532	Camphor	4.5	tR, MS
12	1553	Linalool	75.0	tR, MS
13	1706	α-Terpineol	0.3	tR, MS
14	1765	Geranyl acetate	3.5	tR, MS
15	1857	Geraniol	1.5	tR, MS
		Total	99.8	

[a]RRI: Relative retention indices calculated against *n*-alkanes; [b]%: calculated from FID data; [c]IM: Identification Method; tR, identification based on the retention times (tR) of genuine standard compounds on the HP Innowax column; MS, tentatively identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

3.2. Antibacterial Activity

The antibacterial properties of essential oils and their constituents play an important role in their utilization. Therefore, in the frame of this work, the antibacterial potential of the oil and its constituents were subjected to various in vitro evaluations. In the present study, the MIC values of Coriander essential oil and (+)-linalool were found as 2500 µg/mL against *B. cereus, P. aeruginosa, E. coli, A. baumannii* and 5000 µg/mL against the pathogen *S. aureus*. For comparison the MIC values of standard antibiotics ciprofloxacin and chloramphenicol were given in Table 2.

Table 2. Antibacterial evaluation of Coriander essential oil (CO) and its main constituent (L) (MIC; µg/mL).

Test microorganisms	CO	L	CRP	CHL
A. baumannii	2500	2500	0.25	32
B. cereus	2500	2500	0.25	4
E. coli	2500	2500	>0.125	2
P. aeruginosa	2500	2500	>0.125	16
S. aureus	5000	5000	8	64

3.3. Synergistic Antibacterial Activity

As a result of our present checkerboard experiment, Coriander essential oil showed synergistic effect with ciprofloxacin against *B. cereus* and *E. coli*, where the resulting FIC values are listed in Table 3. Coriander essential oil and chloramphenicol combination showed synergistic effects against *S. aureus* and additive effect against *A. baumannii*. However, Coriander essential oil and chloramphenicol combinations resulted in indifferent effect against *B. cereus* and *E. coli* where the FIC values are given in Table 4. Additionally, the main compound linalool showed synergistic effects with ciprofloxacin combination against *B. cereus* and *E. coli* and with chloramphenicol against *S. aureus* as seen in Tables 5-6, respectively. However, linalool and standard antibiotics combinations resulted in indifferent effect against the tested bacteria.

Table 3. Coriander oil (CO) combinations (comb) with ciprofloxacin (CPR) (µg/mL).

Test microorganisms	CO (Alone)	CO (Comb)	CRP (Alone)	CRP (Comb)	∑FIC	Results
A. baumannii	2500	78.125	0.25	0.25	1.031	Indifferent
B. cereus	2500	312.5	0.25	0.0625	**0.375**	**Synergistic**
E. coli	2500	39.062	0.125	0.0078	**0.078**	**Synergistic**
S. aureus	5000	5000	8	4	1.5	Indifferent

Table 4. Coriander oil (CO) combination (comb) with chloramphenicol (CHL) (µg/mL).

Test microorganisms	CO (Alone)	CO (Comb)	CHL (Alone)	CHL (Comb)	∑FIC	Results
A. baumannii	2500	1250	32	16	**1.0**	**Additive**
B. cereus	2500	78.125	4	4	1.031	Indifferent
E. coli	2500	78.125	2	4	2.031	Indifferent
S. aureus	5000	1250	64	8	**0.375**	**Synergistic**

Table 5. (+)-Linalool (L) combination with ciprofloxacin (CPR) (µg/mL).

Test microorganisms	L (Alone)	L (Comb)	CRP (Alone)	CRP (Comb)	∑FIC	Results
A. baumannii	2500	78.125	0.25	0.25	1.031	Indifferent
B. cereus	2500	625	0.25	0.031	**0.375**	**Synergistic**
E. coli	2500	78.125	0.125	0.0078	**0.093**	**Synergistic**
S. aureus	5000	78.125	8	8	1.015	Indifferent

Table 6. (+)-Linalool (L) combination with chloramphenicol (CHL) (µg/mL).

Test microorganisms	L (Alone)	L (Comb)	CHL (Alone)	CHL (Comb)	∑FIC	Results
A. baumannii	2500	2500	32	16	1.5	Indifferent
B. cereus	2500	78.125	4	8	2.031	Indifferent
E. coli	2500	78.125	2	2	1.031	Indifferent
S. aureus	5000	1250	64	8	**0.375**	**Synergistic**

4. DISCUSSION and CONCLUSION

The linalool enantiomers of various plants including Coriander essential oil were previously reported from our group (Özek *et al*., 2010). According to a previous work of Ebrahimi *et al*. (2010) linalool (40.9−79.9%), neryl acetate (2.3−14.2%), *γ*-terpinene (0.1−13.6%) and *α*-pinene (1.2−7.1%), were reported as the main constituents of Coriander essential oil obtained from different areas in Iran. In other study, linalool (69.8%), *α*-pinene (5.4%), *γ*-terpinene (5.3%) and camphor (5.2%) were found as main constituents for Coriander essential oil (Delaquis *et al*., 2002). Recent works of the chemical compositions of Coriander essential oil by Soares *et al*. (2012) and Scazzocchio *et al*. (2015) reported linalool concentrations of 54.6% and 48.4%, respectively.

In part of our previous work, the antibacterial activities of (-)- and (+)-linalool enantiomers showed no significant effects except against the methicillin-resistant *S. aureus* (200 µg/mL) pathogen (Özek *et al*., 2010). In contrary, in a recent study, linalool was evaluated against five different *A. baumannii* strains where MIC values were 2-8 µL/mL (eq. to 1.74 - 6.94 mg/mL) (Alves *et al*., 2016). Also, Delaquis *et al.* (2002) tested in an earlier antibacterial combination work, high concentrations of linalool fractions (65.1-97.7%), which was effective (0.04-0.47%, v/v) against the tested 5 microbial strains, where *S. aureus* (0.08-0.4%, v/v) was also present. Silva *et al*. (2011) also reported the inhibition results of Coriander essential oil against *A. baumannii* 2/10 with a value of MIC=0.08 mg/mL; for *B. cereus* ATCC 11778, MIC=0.08 mg/mL; for *E. coli* ATCC 25922, MIC=0.17 mg/mL; for *P. aeruginosa* ATCC 27853, MIC=1.42 mg/mL; and for *S. aureus* ATCC 25923, MIC=0.17 mg/mL, respectively. Additionally, the essential oil mode of action was reported through the membrane. In another study by Bazargani and Rohloff (2015), the Coriander essential oil was reported as inhibitory against *S. aureus* CCUG 4151 (MIC=0.68 mg/mL) and against *E. coli* CCUG 17620 (MIC=1.31 mg/mL). Furthermore, Coriander essential oil showed better antibacterial activity compared with chloramphenicol and ciprofloxacin using by disc diffusion method. The antibacterial activity results showed that Coriander essential oil was effective against *S. aureus* with a diameter of 19 mm inhibition, where it can also be used to control *P. aeruginosa* and *E. coli*, however with a relative less inhibition rate in an agar-based environment (Singh *et al*., 2002).

Pathogens such as Gram-positive and Gram-negative (*A. baumannii, S. aureus, B. cereus, E. coli, P. aeruginosa* etc.) are commonly found in water, soil, animals and human. In particular, food borne and spoilage microorganisms are difficult to handle in the treatment of multiple drug resistance. When standard antibiotics and essential oils are used in combination, synergistic activity can increase effectiveness (Van Vuuren *et al*., 2009; Stanojevic *et al*., 2010; Alves *et al*., 2016; Duarte *et al*., 2012; Toroğlu, 2011; Mazumder, 2014).

Previous published work on the synergistic activity by Checkerboard of Coriander essential oil and antibiotic combinations resulted in a synergistic effect with chloramphenicol, ciprofloxacin, tetracycline and gentamicin against *A. baumanii* LMG 1025 and LMG 1041 strains (Duarte *et al*., 2012). An additive effect was also reported for Coriander essential oil combined with gentamicin against the tested bacteria, except for *A. baumanii* 93641 with FICI value of 0.25 (Scazzocchio *et al*., 2015). The study by Toroglu *et al*. (2011) where Coriander essential oil was combined with Ceftriaxone resulted in an increase of antibacterial activity against *S. aureus*. Additionally, the combination of Coriander essential oil with gentamicin, cephalothin and ceftriaxone resulted in the decrease of the antimicrobial activity against *S. aureus* Cowan 1 and *E. coli* DM strains.

Mazumder *et al*. (2014) observed differences in survival rates of essential oils and bacteria. *E. coli, S. aureus* and *P. aeruginosa* antibiotics were found to be more susceptible to growth inhibition of antibiotics when Coriander essential oil was added compared to the inhibition

zones on nutrient agar plates. Ciprofloxacin and streptomycin were reduced in the combinations with Coriander essential oil further confirming synergistic activity.

As an overall result, it was observed that generally the inhibitory concentrations against a wide spectrum of pathogens were decreased with oil and antibiotic combinations to contribute also to lower the microbial resistance as well as overall toxicity. The previously reported antimicrobial study (Silva *et al*., 2011) also confirmed the mode of action of Coriander essential oil against the pathogens.

This study was performed to investigate the antimicrobial activities of Coriander essential oil and main constituent linalool against human and food pathogens. To the best of our knowledge the combination of the essential oil and the major components (+)-linalool with selected common food and human pathogens were performed in this present study. Moreover, essential oil and linalool had been shown synergistic antibacterial activities combined with standard antibiotics chloramphenicol and ciprofloxacin on particular standard pathogenic bacterial strains. Our results were also in accordance with current literature results. It can be concluded that compared with information in the literature, our Coriander and linalool inibitory results were in agreement.

These present results showed that the particular combinations could be an alternative treatment for multidrug resistance in various microbial infections in particular originating from food sources, which has to be confirmed by in-depth studies.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Gözde Öztürk: Investigation, Methodology, Formal Analysis and Writing original draft. **Gamze Göger:** Investigation, Methodology, Formal Analysis and Writing original draft. **Fatih Demirci:** Supervision and Writing original draft. **Betül Demirci:** Supervision and Critical reading.

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Research Article

Phytochemical screening and in vitro antioxidant activities of *Mentha suaveolens* Ehrh. extract

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Abstract: Within the framework of the valorization of the medicinal and aromatic plants of Morocco, we were interested during this study in the characterization and the phytochemical identification of some secondary metabolites present in *Mentha suaveolens* Ehrh. and the evaluation of the antioxidant activity of this species using four methods: DPPH free radical scavenging test, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, (ABTS) radical cation scavenging test, FRAP test measuring antioxidant power, and β bleaching-carotene. *Mentha suaveolens* is a species belonging to the Lamiaceae family, harvested in southern Morocco, and widely used in traditional medicine for its biological properties attributed mainly to phenolic compounds. Just as they protect plants, secondary metabolites also have a protective role in the human body and are therefore beneficial to our health. They are attributed, in particular, with antioxidant, anti-inflammatory, and antibacterial properties. In this regard, characterization and identification tests of secondary metabolites revealed the presence of alkaloids, flavonoids, catechic tannins, and terpenes in this plant, whereas, the aerial parts of this species are devoid of coumarins, cyanogenic compounds, saponins and free quinones. The quantification of the phenolic compounds gave high contents, with contents of total phenols (54.75 ± 5.62) µg GAE/mg, total flavonoids (32.41 ± 0.41) µg QE/mg, and total condensed tannins (27 ± 1) µg CE/mg. In addition, the results obtained show that *Mentha suaveolens* extract has stronger antioxidant activity using the β-Carotene method with IC50 (0.24 ± 0.06) mg/mL against IC50 (0.021 ± 0.001) mg/mL for the standard antioxidant by the DPPH free radical scavenging test.

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1. INTRODUCTION

Aromatic plants currently present a reliable source of active ingredients known for their therapeutic properties, in particular, antioxidant activity (Saber *et al*., 2021). In this context, a study recently published under the theme, of ethnobotanical, phytochemical, and antioxidant study of fifty medicinal and aromatic plants, the results obtained can be considered as a source of information for scientific research in the field of pharmacology and phytochemistry. With a

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view to finding new bioactive compounds (Afrokh *et al*., 2023). In addition, the secondary metabolites from plants have a natural antioxidant power protecting the human body from free radicals, preventing oxidative stress and associated diseases. For these reasons, they play a very important role in health care (Yao *et al*., 2004). Medicinal plants, therefore, constitute a precious heritage for humanity and can be used in several fields in addition to the therapeutic field, namely the fields of food, cosmetics, perfumery, etc.

In Morocco, aromatic and medicinal plants (AMPs) as natural resources have been a focus of interest in recent years for the national economy. It is one of the richest countries in the world in terms of its diversity: 4200 species of plants, 800 of which are endemic, of which 382 species are known for their medicinal and/or aromatic use (Hmamouchi, 1999; Jamila & Mostafa, 2014). The genus Mentha is one of the important elements of the family Lamiaceae; it is represented by 19 species and 13 natural hybrids (El-Kashoury *et al.*, 2015). Among its species, include *Mentha suaveolens*, which is located in North Africa, Europe, America, and Japan (Sutour *et al.*, 2010).

Mentha suaveolens (MS) or the round-leaved mint has long been known as *M. rotundifolia* (L.) Huds (Harley & Brighton, 1977), is an herbaceous perennial, with a characteristic minty smell, pubescent with a quadrangular stem, with oval leaves, whitish green, covered with network wrinkles. The inflorescences are slender, elongated spikes of small white or slightly pinkish flowers (J. Bellakhdar, 2006). Its flowering takes place from July to September (Kumar, Mishra, Malik, & Satya, 2011). This plant has a wide range of benefits: antispasmodic, analgesic, anti-inflammatory, antimicrobial, acetylcholinesterase, choleretic, carminative, tonic, hypotensive, sedative, stomachic, insecticidal, hepatoprotective, monoamineoxidase inhibitor, it is also applied in the treatment of digestive problems, influenza, respiratory diseases, rheumatism, irritation, skin diseases, nausea, bronchitis and anorexia (Bellakhdar, 1996; Božović, Pirolli, & Ragno, 2015; Karousou *et al.*, 2007; Moreno *et al.*, 2002). In folk medicine, Suaveolens mint products have been used as a carminative (Bellakhdar, 2006). In addition, this species is widely used in the Maghreb to prepare a special pancake that is eaten in winter against the cold. The plant is also used to filter melted butter, which gives it flavor and improves its preservation (Bellakhdar, 2006). The fragrant mint with round leaves contains an essential oil (EO) which can belong to different chemotypes according to the places of harvest. The main components most often encountered are piperitone oxide, piperitone oxide, piperitone, pulegone, neo-isopulegone, carvone, dihydrocarvone (Bellakhdar, 2006).

The main objectives of this study were to characterize the different secondary metabolites present in the aerial parts of MS, which are widely used in southern Morocco for therapeutic purposes, to quantify the levels of phenolic compounds, and to evaluate the antioxidant activity of the methanol extract by following four different methods.

2. MATERIAL and METHODS

2.1. Chemicals and Plant Material

All solvents were of analytical or HPLC grade and purchased from Professional Labo (Casablanca, Morocco). The chemical reagents used in this work are classified as follows: 2,2'-azino-bis (3-ethyl benzthiazoline-6-sulphonic acid), 2,2-diphenyl-1-pycridazil (DPPH 90%), β-Carotene, aluminum chloride (AlCl3), ferric chloride hexahydrate (FeCl3,6H2O), antimony chloride (SbCl3), Iodoplatinate, Dragendorff, Mayer, Neu's reagent, potassium persulphate (K2S2O8), potassium ferricyanide K3Fe(CN)6, Folin-Ciocalteau's phenol reagent, sodium carbonate (Na2CO3), sodium hydroxide (NaOH), Gallic acid, Ascorbic acid, Quercetin, Catechin, and Vanillin were sourced from Professional Labo (Casablanca, Morocco).

Mentha suaveolens was collected near the town of Er-rich, located 65km from the province of Errachidia (Latitude: N: 32° 15'33.691", Longitude: O: 4° 29'43.544" and Altitude: 1321 meter) in full bloom during September.

2.2. Phytochemical Screening

With the aid of qualitative characterization reactions, phytochemical screening assays look for various families of secondary metabolites that are present in this plant's aerial portion. These processes rely on precipitating or staining events and use reagents specific to each chemical family (Hagerman AE, 2000). Our study focused on the following compounds: alkaloids, coumarins, cyanogenic compounds, flavonoids, tannins, terpenes, saponins, and quinones.

2.2.1. *Saponosides*

In a beaker, 100 mL of distilled water is added to a quantity of 1 g of dry plant material, then the solution is boiled for 30 min. After cooling, the solution is filtered, and the filtrate is adjusted to 100 mL with distilled water. A series of 1 to 10 mL of filtrate is placed in 10 test tubes, the final volume being readjusted to 10 mL with distilled water. A violent and horizontal agitation was made for 15 seconds for each tube. After 15 minutes of rest, the height of residual foam is measured (in cm) in each tube and whether it is close to 1 cm in the Xth tube. The presence of saponins is indicated by a foam index greater than 100. The latter is calculated according to the following relationship (Alilou et al., 2014).

$$I = \text{The height of the foam in the } X^{th} \text{ tube x } 10/0.0X$$

2.2.2. *Tannins*

A quantity of 1.5 g of dry plant material was placed in 10 mL of 80% methanol and stirred for 15 minutes then filtered on filter paper. A few drops of 1% $FeCl_3$ are added to the methanolic extract already prepared. In the presence of gallic and ellagic tannins, a blue-black coloring is observed, whereas in the presence of catechin tannins, this coloring is greenish brown (Alilou et al., 2014).

2.2.3. *Free quinones*

A quantity of 0.5 g of the dry plant material is placed in 5 mL of petroleum ether. After a few minutes of stirring, the mixture is left to stand for the whole day. After filtering this mixture, it is concentrated using a rotavapor. The change in color of the aqueous phase to yellow, red, or purple after adding a few drops of NaOH (1/10), testifies to the presence of quinones (Alilou *et al.*, 2014).

2.2.4. *Terpenoids*

To a quantity of 1 g of the crushed plant material, 5 mL of hexane was added, followed by sonication for 15 minutes. After stirring for 30 min and filtration, migration of the filtrate was carried out on a preparatory silica gel plate, the solvent used is benzene. After migration, the plate is sprayed with antimony chloride (prepared in chloroform) and then placed in an oven at 110° C. for 10 min. Any fluorescence at 365 nm indicates the presence of terpenoids (Alilou *et al.*, 2014).

2.2.5. *Coumarins*

Coumarins are detected by two different tests:

*The first test: Detection test A quantity of 2 g of crushed dry plant material is placed in 10 mL of chloroform. Everything is heated for a few minutes and then filtered using filter paper. The migration of this solution was made on a thin layer in the solvent: toluene/ethyl acetate (93/7). After drying under a ventilated hood, the revelation was made using NH_3 vapor under UV at 365 nm (Alilou *et al.*, 2014).

*The second test: Confirmation test 1 g of crushed dry plant material is weighed and placed in a test tube, in the presence of a few drops of water, the tube is covered with filter paper soaked in diluted NaOH. The whole is placed in a boiling water bath for a few minutes. The filter paper is then examined under UV light at 365nm. Any yellow fluorescence indicates the presence of coumarins (Alilou et al., 2014).

2.2.6. *Cyanogenic compounds*

A quantity of 1g of fresh plant material is wetted with a few drops of chloroform $CHCl_3$ in a test tube where a strip of filter paper impregnated with sodium picrate is inserted. The whole is heated in a water bath at 35°C. for 3 hours. A red turn of the strip after the production of HCN indicates the presence of cyanogenic compounds (Alilou *et al*., 2014).

2.2.7. *Alkaloids*

The presence of alkaloids has been demonstrated by three different tests, which have a qualitative purpose: The Iodoplatinate, Dragendorff, and Mayer tests (Alilou *et al*., 2014), because some alkaloids may be sensitive to certain tests and not detectable by others.

*Preparation of methanolic extracts: Two grams of plant material, dry and ground, is added to 100 mL of 80% methanol. After sonication for 15 min and stirring overnight, the extracts are filtered and evaporated to dryness using a rotary evaporator. The residues are taken up in a few ml of pure methanol. These extracts are subjected to the following two tests:

*Iodoplatinate test: The methanolic extract to be tested is deposited on a thin layer (silica plate) the chromatogram is developed in the following solvent: (AcEt/MeOH/NH_4OH) (9/1/1), then dried under a fume hood. The migration bands are identified and delimited under UV light at 365 nm. Application of the Iodoplatinate reagent by spraying reveals the presence of alkaloids. These show up as a blue to purple color on the chromatogram.

*Dragendorff test: It is based on the same principle as the Iodoplatinate test, except that it is revealed by spraying with Dragendorff reagent. The appearance of bright orange spots on the chromatogram indicates the presence of alkaloids.

*Mayer test: To a quantity of 0.5 g of crushed dry plant material, 15 mL of ethanol (70%) is added and in order to destroy the cell walls and release all the constituents that bathe in the vacuole, sonication is carried out for 15 min. Then, the extracts are left under magnetic stirring overnight. After complete decantation, filter through filter paper. The extract is evaporated to dryness in the rotavapor. The residue recovered in a few ml of HCl (50%) is then transferred to two test tubes; one is used as a control and to the other Mayer's reagent is added. The appearance of a white precipitate reflects the presence of alkaloids.

2.2.8. *Flavonoids*

One gram of dry powder plant material is extracted with 20 mL of 80% MeOH. After stirring for 15 min and another 15 min of sonication, the extracts are filtered and subjected to TLC, the migration solvent being glacial acetic acid/H_2O (15/85). Visualization is done at 365 nm after spraying with Neu's reagent (2-aminoethyl diphenyl borate) at 1% in pure MeOH. (Dohou *et al*., 2003).

2.3. Quantitative Determination Assays

2.3.1. *Total phenolic content*

The quantification of the phenolic content of the methanol extract was determined using the Folin- Ciocalteu method (Singleton *et al*., 1999). Briefly, 100 µL of extract or standard antioxidant (gallic acid (GA)) was mixed with 500 µL of the Folin-Ciocalteu reagent (10 times diluted in distilled water). After 2 minutes of incubation, 2.00 mL of the 20% Na_2CO_3 solution was added. The mixture was left to settle for 30 minutes at room temperature in the dark, and

the absorbance is read at 765 nm using LLG-uniSPEC 2 Ultraviolet–Visible spectrophotometer, against a blank without extract. The produced solutions' optical densities were utilized to draw the GA calibration curve. Total phenolic content was calculated from the linear regression equation (**y** = 0,004x + 0,089 with a correlation coefficient R^2 = 0.982) of gallic acid and expressed as micrograms of gallic acid equivalent per one milligram of extract (µg GAE/mg of extract). All measurements are performed in triplicate.

2.3.2. *Total flavonoids content*

The total flavonoid content of the extract was determined by the colorimetric method using aluminum trichloride as described by (Quettier-Deleu *et al*., 2000). Briefly, 1.00 mL of plant extract or quercetin standard solution was mixed separately with 1.00 mL of 2% aluminum chloride. After 10 minutes in the dark and at room temperature, the absorbance is read at 430 nm with a spectrophotometer (LLG-uni spectrophotometer). The total flavonoid concentration is calculated from the regression equation of the calibration range established with standard quercetin prepared in methanol (y=0.006x+0.079 with a correlation coefficient R^2=0.991). The result is expressed in micrograms of quercetin equivalent per gram of extract (µg QE/mg of extract). All tests are repeated three times.

2.3.3. *Total condensed tannin content*

The quantification of condensed tannins (proanthocyanidins) in the extract using the method of Sun *et al.* in 1998 (Sun, Ricardo-da-Silva, & Spranger, 1998). 50 µL of the sample or diluted standard was mixed with 3 mL of a 4% vanillin-methanol solution and 1.5 mL of 37% hydrochloric acid was added. 15 minutes were given for the mixture to stand. at room temperature. The absorbance was then measured at 500 nm against a water/methanol mixture (v/v) as a blank. A calibration curve was produced in parallel under the same operating conditions using catechin (C). The total condensed tannin contents are calculated from the regression equation of the calibration range established with catechin (y=0.001+0.025, R^2=0.996, where y was the absorbance and x was the concentration). The result is expressed in micrograms of catechin equivalent per gram of extract (µg CE/mg of extract). All tests are replicated three times.

2.4. Evaluation of Antioxidant Activity

2.4.1. *DPPH free radical scavenging activity*

The free radical scavenging capacity of the methanolic extract was determined using the stable free radical, 2,2 diphenyl-1-picryhydrazyl ($DPPH^{\bullet}$) according to the method explained by (Loo, Jain, & Darah, 2008). Briefly, 1.80 mL of a 0.004% methanol DPPH solution is added to 0.20 mL of various concentrations of methanol extract or standard and allowed to stand in the dark for 30 minutes for the reaction to occur. The absorbance of the mixture is measured at 517 nm using a spectrophotometer (LLG-uni spec2spectrophotometer). The IC50 value (50% inhibitory concentration) was calculated and obtained from the linear regression (Molyneux, 2004). A low IC_{50} value indicates high antioxidant activity. The experiment was done in triplicate.

2.4.2. *Ferric reducing antioxidant power (FRAP)*

The reducing power of iron (Fe^{3+}) in the extract is determined according to the method described by Oyaizu (Oyaizu, 1986). One milliliter of the extract at different concentrations is mixed with 2.5 mL of a 0.2 M phosphate buffer solution (pH= 6.6) and 2.5 mL of a solution of $K_3Fe(CN)_6$ at 1%. The whole is incubated in a water bath at 50°C for 20 min, then 10% trichloroacetic acid (2.5 mL) is added to stop the reaction, and the tubes are centrifuged for 10 min at 3000 rpm. An aliquot (2.5 mL) of supernatant is combined with 2.5 mL of distilled water and 0.5 mL of an aqueous solution of ($FeCl_3$, $6H_2O$) at 0.1%. The reading of the absorbance of the reaction medium is done at 700 nm against a similarly prepared blank, replacing the extract

with distilled water which makes it possible to calibrate the device (LLG-uni spec2spectrophotometer). The positive control is represented by a solution of a standard antioxidant whose absorbance was measured under the same conditions as the samples.

2.4.3. *ABTS radical cation scavenging assay*

The solution of ABTS radical cation ($ABTS^{\bullet+}$) is prepared by mixing 2 mM of an ABTS with 70 mM of a solution of potassium persulfate (v/v). Before usage, the mixture is stirred for 24 hours in the dark and at room temperature. The solution was then diluted with methanol to achieve an absorbance of 0.700±0.02 at 734 nm. 2 mL of this solution, 200 µL of extract or positive control are added, and the absorbance obtained after 30 min at 734 nm is noted (Müller, Fröhlich, & Böhm, 2011).

2.4.4. *β-Carotene bleaching test*

The antioxidant activity of the aqueous solution was determined by a β-carotene/linoleic acid system (Bougatef *et al.*, 2009). Briefly, in a round bottom flask, we put 40 µL of linoleic acid, 1 mL of β-carotene solution (2 mg/mL in chloroform), and 400 µL of Tween 20. A stream of nitrogen was used to evaporate the chloroform from the mixture. Then, distilled water (100 mL) was slowly added to the residue and vigorously stirred to give a stable emulsion. A 2.5 mL aliquot of this emulsion was added to 500 µL of methanolic solution of MS prepared at different concentrations. 500 µL of distilled water was added to the control reaction mixtures. At 470 nm, absorbance was immediately measured. After 120 minutes, the absorbance of the tubes was measured in a water bath at 50°C.

3. RESULTS and DISCUSSION

3.1. Phytochemical Screening

The results of the screening tests carried out on the aerial parts of MS, are shown in Table 1. The characterization tests made it possible to identify the main chemical groups contained in the aerial parts of MS such as catechin tannins, flavonoids, terpenes, and alkaloids. On the other hand, the other families such as free quinones, coumarins, saponosides, cyanogenic compounds, and Gallic tannins were not detected (Figure 1).

Figure 1. Phytochemical screening of *Mentha suaveolens*

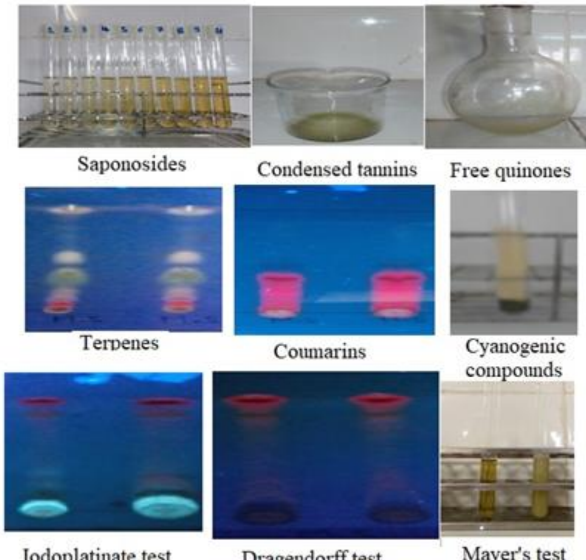

Table1. Results of phytochemical screening of aerial parts of *Mentha suaveolens*

	Secondary metabolites		Solvent	Reagent/developer Without or with UV 365nm	Observations	Results
Mentha suaveolens	Saponosides		Eau distillée	Foam indices	Foam height 0.6	-
	Condensed tannins	Catechin	Methanol	Iron chloride ($FeCl_3$) à 1%	Greenish brown	++
		Gallic			-	-
	Free quinones		Petroleum ether	NaOH 0,1 N	-	-
	Terpenes		Benzene	Antimony chloride	Sky blue	+++
	Coumarins		Toluene/ethyl acetate: (93/7)	NH_3 vapors	-	-
	Cyanogenic compounds		Chloroform	Sodium Picrate	Yellow	-
	Alkaloids		AcEt/MeOH / NH_4OH : (9/1/1)	Dragendorff	Orange to brown	++
				Iodoplatinate	Blue to purple	
			Methanol	Mayer	Yellowish white	
	Flavonoids		Glacial acetic acid/water: 15/85	Neu reagent	Light blue, Yellow and Dull yellow	++

+++ : Strongly test
++ : Positive average test
+ : Low positive test
- : Negative test

The effective presence of certain secondary metabolites and the absence of others does not exclude the therapeutic properties of these plants (Kabran, 2011). These compounds are known for their bioactive properties. Flavonoids are very effective and non-toxic antioxidants; they are antispasmodic, anti-ulcer, anti-secretory, anti-allergic, anti-diarrheal, and anti-inflammatory, and they protect against cancer and cataracts (Bimakr *et al.*, 2011; Bruneton, 2009). There are pharmacological activities associated with alkaloids such as enhancement of cardiac activity, excitation of the central nervous system and symptomatic nerves, and stimulation of blood circulation (Kabran, 2011). Certain diseases may also be treated with these plants due to their alkaloids (N'Guessan *et al.*, 2009). Tannins show the properties of vitamin D, they could be utilized to support blood vessels and aid in the body's absorption of vitamin C. (Kabran, 2011). As for terpenes, they are used as additives in the food and cosmetic industries (Tsao & Coats, 1995), and numerous of them have biological activities: anti-carcinogenic, antimicrobial, anti-inflammatory, insecticidal (Murakami *et al.*, 2004), anesthetic and antihistamine (mono and sesquiterpenes), diuretic (β-eudesmol) *(Veličković et al.*, 2003; Hsiou, 2000), neuroprotective (α-terpinene, γ-terpinene, and trans-caryophyllene) (Chang, Kim, & Chun, 2007). Anti-tumor and cytotoxic properties of diterpenes (taxol) and the antioxidant activities attributed above all to phenolic diterpenes (Gill, 1993) may also be mentioned. The presence of coumarins explains the antifungal (Kandaswamy & Raveendiran, 2014), antibacterial (Bhat, Al-Omar, & Siddiqui, 2013), antiviral (R. W. Fuller, 1994)), antimalarial (Yang *et al.*, 1992), anti-inflammatory (Bhat *et al.*, 2013; Chang *et al.*, 2007; García-Argáez *et al.*, 2000; Gill, 1993; Hiermann, Schramm, & Laufer, 1998; Kandaswamy & Raveendiran, 2014; Milcent & Chau, 2003; Murakami *et al.*, 2004; Fuller, 1994); Tsao & Coats, 1995; Veličković *et al.*, 2003; Hsiou, 2000; Yang *et al.*, 1992), anti-tumor (Fujioka *et al.*, 1999; Kofinas *et al.*, 1998) and anticoagulant effect (Egan, 1990).

3.2. Estimation of The Phenolic Compound Content

The results of the assay of the phenolic compounds in the methanolic extract of the aerial parts of MS are grouped in Table 2.

Table 2. Contents of phenolic compounds in the aerial parts of *Mentha suaveolens.*

The phenolic compounds	Total phenolic (µg GAE /mg)	Total flavonoids (µg QE/mg)	Total condensed tannin (µg CE/mg)
Content	54.75 ± 5.62	32.41 ± 0.41	27 ± 1

According to our results cited in the table above, it appears that the content of total phenols in our methanolic extract of MS (54.75 ± 5.62) µg GAE /mg of extract) is lower than those of Salhi et al. 2017(145±7.48) µg GAE /mg (Salhi et al., 2017), whereas, the content of total flavonoids in our methanolic extract of MS (32.41 ± 0.41) µg QE/mg of extract) is closer to those of Salhi *et al.* (2017) (30.57±2.13) µg QE/mg(Salhi *et al.*, 2017) and also higher than those of Bichra *et al.* (2013) (0.1 ± 0.02) CE µg /mg and (0.3 ± 0.08) CE µg /mg Dry Matter (DM) for the aqueous and phenolic extract, respectively (Bichra, El-Modafar, El-Abbassi, Bouamama, & Benkhalti, 2013). Additionally, the condensed tannin content for our methanolic MS extract is (27 ± 1) µg CE/mg extract.

3.3. Evaluation of Antioxidant Activity

In accordance with the results gathered in Table 3, it is important to emphasize that the methanolic extract of *Mentha suaveolens* has a slightly moderate antioxidant power for the four tests at the rate of IC50: (0.29 ± 0.02) mg/mL, (0. 25 ± 0.03) mg/mL and (0.28 ± 0.01) mg/mL, (0.24 ± 0.06) mg/mL for the tests: $DPPH^{\bullet}$ Free radical scavenging activity, Ferric Reducing Antioxidant Power (FRAP), $ABTS^{\bullet+}$ Radical Scavenging Test and Whitening of the β-

Carotene, respectively but remains less important than the standard antioxidant, ascorbic acid for the four tests at the rate of IC50: (0.021 ± 0.001) mg/mL, (0.022 ± 0.004) mg/mL, (0.031 ± 0.002) mg/mL, (0.027 ± 0.005) mg/mL, respectively for the same tests mentioned above in the same order. By comparing our results with those of the literature for the DPPH test, it appears that the value obtained for the methanolic extract of MS is in good agreement with those of Madiha *et al*. (2012) (Bichra & Benkhalti, 2012), particularly in terms of inhibition percentages and higher than Kasrati *et al*. (2017) (19.51 ± 0.04 µg/mL), especially for *Mentha suaveolens*.

According to our results for the FRAP test, we can suggest that the reducing power of our extract is probably due to the presence of hydroxyl group in the phenolic compounds which can serve as an electron donor. Therefore, antioxidants are considered reducers and inactivators of oxidants (Siddhuraju & Becker, 2007). To our knowledge, no study has been carried out on the methanolic extract of MS using the $ABTS^{\bullet+}$ cation radical reduction test.

In addition, according to a study conducted by Kasrati *et al*. (2017) (Kasrati *et al*., 2017) on *Mentha suaveolens* subsp timija (Briq.) Harley using the β-Carotene bleaching test, it turns out that their extract (IC_{50}= 64.92 ± 0.90 µg. mL^{-1}) has an antioxidant effect more important than ours.

Table 3. Antioxidant activity of MS *extract* using DPPH, ABTS, FRAP and β-Carotene bleaching methods.

Sample/Standard	Antioxidant activity tests			
	DPPH IC_{50}(mg/mL)	FRAP IC_{50}(mg/mL)	ABTS IC_{50}(mg/mL)	β-Carotene bleaching IC_{50}(mg/mL)
Methanolic extract	0.29 ± 0.02	0.25 ± 0.03	0.28 ± 0.01	0.24 ± 0.06
Ascorbic acid	0.021 ± 0.001	0.022 ± 0.004	0.031 ± 0.002	0.027 ± 0.005

4. CONCLUSION

This work concerns the phytochemical study of a species belonging to one of the major plant families serving as a framework for evaluating the presence of certain typical secondary metabolites. Just as they protect plants, secondary metabolites also have a protective role in the human body and are therefore beneficial to our health. They are attributed in particular with antioxidant, anti-inflammatory, and antibacterial properties. In this regard, characterization and identification tests of secondary metabolites revealed the presence of alkaloids, flavonoids, catechic tannins, and terpenes in this plant. But, the aerial parts of this species are devoid of coumarins, cyanogenic compounds, saponins, and free quinones. The quantification of phenolic compounds gave high contents, with contents of total phenols (54.75 ± 5.62) µg GAE /mg, total flavonoids (32.41 ± 0.41) µg QE/mg, and total condensed tannins (27 ± 1) µg CE/mg. In addition, the results obtained show that the extract of this plant has a stronger antioxidant activity for the β-Carotene method with IC_{50} (0.24 ± 0.06) mg/mL against IC_{50} (0.021 ± 0.001) mg/mL for the standard antioxidant by the test DPPH radical scavenging. However, the results of this study showed that the extract of the aerial parts of this plant has antioxidant capabilities that could act as free radical scavengers or inhibitors or possibly act as a natural antioxidant.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Moha Afrokh: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft**. Kamal Boumhara:** Investigation, Resources**. Khalid Chatoui:** Investigation, Resources, Visualization, Methodology**. Saida Tahrouch and Abdelhakim Hatimi**: Methodology, Supervision, and Validation. **Hicham Harhar** and **Mohamed Tabyaoui**: visualization, editing the original draft.

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Investigation of *In Vitro* antiproliferative activity properties of *Spartium junceum* L. (Spanish broom) against MDA-MB-231 and HepG2 cancer cell lines

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Abstract: Cancer is among the top global public health burdens leading to millions of deaths each year. The study aims to investigate the antiproliferative effect of *Spartium junceum* L. flowers on different cancer cell lines. The ethanolic extract of the flowers was prepared in the present study. Phytochemical analysis of the plant extract revealed the presence of several phenolic compounds such as cinnamic acid and its derivatives (chlorogenic, *p*-coumaric, ferulic acids), protocatechuic acid, epicatechin and luteolin. This extract was tested against human breast (MDA-MB-231) and liver (HepG2) cancer cell lines to find out its antiproliferative activity. It was determined that the extract was effective against both cell lines with IC_{50} values of 2.37 ± 0.47 and 0.98 ± 0.01 µL/mL for MDA-MB-231 and HepG2, respectively. Particularly, the extract was found to be more effective in the liver cancer cell line than the breast cancer cell line. All these obtained findings led us to believe that this medicinal plant could be a promising antiproliferative agent candidate for the treatment of human liver and breast cancers.

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1. INTRODUCTION

Cancer is one of the significant global public health burdens leading to millions of deaths worldwide every year. This fatal disease is characterized by the transformation of normal body cells into abnormal ones that divide at an uncontrollable rate and can invade other parts of the body causing metastasis (Wu *et al*., 2019).

Carcinogenesis can affect any part of the body, and cancer is named after the part of the body in which it originated (Sahayarayan *et al*., 2021). Among the cancer types, hepatocellular carcinoma (HCC) is the second cause of cancer mortality and the rate of its incidence has continuously increased day by day (Llovet *et al*., 2022). Similarly, breast cancer is the most commonly diagnosed cancer in women and is responsible for nearly 900 thousand deaths per

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year (Allugunti, 2022). According to the World Health Organization's cancer report, there were nearly 10 million deaths in 2020 (World Health Organization, 2023).

The main components of cancer treatment include radiation therapy, surgery, and chemotherapy (van den Boogaard *et al*., 2022). Chemotherapy is an effective treatment option that increases the survival rate of people suffering from cancer. In this method, malignant cells that can harm healthy cells are killed by strong chemicals (Dennis *et al*., 2022). However, modern chemotherapeutics are associated with severe unpleasant side effects such as neurotoxicity, nephrotoxicity, cardiotoxicity, hepatotoxicity, and ototoxicity. Furthermore, the resistance of tumor cells to specific chemotherapeutics is one of the significant problems of chemotherapy (van den Boogaard *et al*., 2022). For all these reasons, there is an urgent need for more research to explore new and safe treatment strategies.

Increasing evidence has shown that some medicinal plants represent an excellent source for screening new and safe chemotherapeutics. The plant-based anti-cancer chemical compounds such as taxol, topotecan, irinotecan, vincristine, vinblastine, and etoposide are used clinically worldwide (Imran & Shahid, 2022).

Spartium junceum L. (Spanish broom) is a flowering perennial medicinal shrub belonging to the Fabaceae family. Flowers of this plant are rich in various secondary metabolites such as flavonoids, saponins, and cytisine-type alkaloids (Nadaf *et al*., 2012; Yeşilada *et al*., 2000a; Rammal *et al*., 2021). In previous studies, the flowers have been found to have anti-ulcerogenic, antitumor, analgesic, anti-inflammatory, antiviral, and antioxidant properties (Yeşilada *et al*., 2000b; Nanni *et al*., 2018; Menghini *et al*., 2006; Duman *et al*., 2019).

In the present work, the antiproliferative activity potential of the ethanolic extract prepared from the flowers of *Spartium junceum* L. was evaluated in different human cancer cell lines: breast adenocarcinoma (MDA-MB-231) and liver hepatocellular carcinoma (HepG2). The study material was chosen considering the presence of the plant's antiproliferative activity in different cell lines in previous reports (Abusamra *et al*., 2015; Cerchiara *et al*., 2012). As a result of the literature review, there is no study in the literature investigating the antiproliferative activity of the plant in the cell lines we selected for this study.

2. MATERIAL and METHODS

2.1. Plant Material and Extraction

Flowers of *Spartium junceum* L. (SJ) were collected from Eğirdir/Isparta on the date of May 19, 2021 (Figure 1). The herbarium sample was authenticated by Assoc. Prof. Gülsen Kendir and deposited in the Herbarium of the Faculty of Pharmacy of Ankara University under voucher number AEF 30711. To prepare the ethanolic extract, 50 g of dried flowers were subjected to maceration with 500 mL of 95% ethanol. The extract was filtered and the filtrate was evaporated to dryness at 36 °C using a rotary evaporator (Heidolph Hei-Vap Rotary Evaporator). At the end of the process, the crude extract remaining in the flask was weighed at 9 g and the yield of the extract was calculated as 18 % and transferred to a vial.

Figure 1. Flower of SJ plant (photograph taken by Fatma Tuğçe Gürağaç Dereli).

2.2. Reagent and Materials

Human liver hepatocellular carcinoma cell line (HepG2) (ATCC® HB-8065TM) and human breast adenocarcinoma cell line (MDA-MB-231) (ATCC® HTB-26TM) were purchased from American Type Culture Collection (ATCC, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified eagle's medium high glucose (DMEM), glutamax and fetal bovine serum (FBS) were purchased from Sigma (USA), Gibco (Life Technologies, USA) or HyClone (USA).

2.3. In Vitro Antiproliferative Activity Studies

MDA-MB-231 and HepG2 cells were cultured in DMEM supplemented with 10% FBS and 1% glutamax under a humidified incubator of 5% CO_2 at 37 °C. When the cells reached the level to be passaged (90% occupancy), the medium in the flask was removed. Cells were washed twice with phosphate buffer saline (PBS). The cells in flasks were passaged using trypsin-EDTA. The cells were seeded into 96-well plates at 5 x 10^3 cells/well density. A stock solution was prepared by dissolving 5 mg of extract in 1 mL of DMEM. After 24 h, the medium was replaced and the cells were exposed to the prepared extract dissolved in DMEM at different concentrations (0.1562, 0.3125, 0.625, 1.25, 2.5, and 5 µL/mL) for 48 h. After this period was completed, the medium in the wells was carefully removed. 5 mg/mL of MTT stock solution was added to each well, and plates were incubated for 2 h. After this period was completed, the medium was removed and 200 µL of dimethylsulphoxide (DMSO) was added to dissolve the formed formazone. It was stirred for half an hour in the dark and at room temperature. The absorbance values were measured with Promega reader device at 560 nm. GraphPad Prism 5 program was used for calculating IC_{50} values.

2.4. Phytochemical Screening

The phenolic profile of the ethanolic flower extract was defined by High-Performance Liquid Chromatography (HPLC) technique. HPLC conditions are presented in Table 1.

Table 1. Chromatographic conditions.

Chromatographic conditions	Time (min.)	A (%)	B (%)
Stok concentration injected to the HPLC system: 0.04 µg/mL Detector: SPD-M 10A vp DAD dedektör (λmax=278nm) Autosampler:SIL–10AD vp System controller: SCL-10A vp Pump: LC-10AD vp Degasser: DGU-14a Column heater: CTO-10 A vp Column: Agilent Eclipse XDB C-18 (250 mm × 4.6 mm), 5 µm Column temperature: 30 °C Mobile phases: A: acetic– water (3:97 v/v), B: methanol Flow rate: 0.8 mL / min. Injection volume: 20 µL	0	93	7
	20	72	28
	28	75	25
	35	70	30
	50	70	30
	60	67	33
	62	58	42
	70	50	50
	73	30	70
	75	20	80
	80	0	100
	81	93	7

3. RESULT and DISCUSSION

3.1. *In Vitro* Antiproliferative Activity Studies

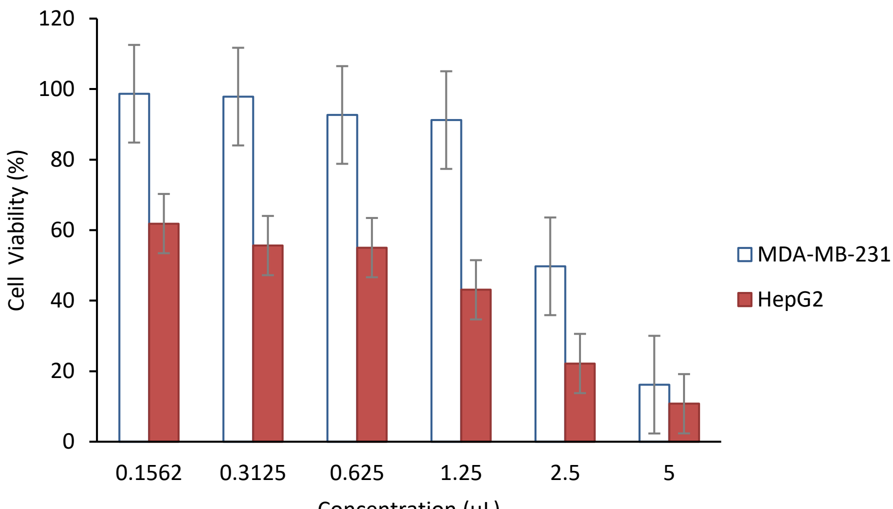
The antiproliferative activities of extract were evaluated against MDA-MB-231 and HepG2 cell lines for 48 h. The experiments were repeated twice. The results and calculated standard deviation values are given in the Table 2.

Table 2. IC_{50} results for SJ in human cell lines.

Compound	IC_{50} (µL/mL)	
	MDA-MB-231	HepG2
SJ	2.37 ± 0.47	0.98 ± 0.01

The effect of the extract in proliferation varies partly depending on the studied concentrations (Figure 2). At 5 µL/mL of the extract, the viability ratio was obtained as 16.18% and 10.77% for MDA-MB-231 and HepG2, respectively. When the amount of extract used was halved (for 2.5 µL/mL), the cell viability rates increased to 49.75% and 22.18% for breast and liver cancer cell lines, respectively. It was observed that the extract used in amounts smaller than 1.25 µL/mL had a similar effect on cell proliferation, and there were no major differences. For the MDA-MB-231 cell line, the cell viability ratio was obtained as 91.22%, 92.67%, 97.88%, 98.69% at 1.25 µL/mL, 0.625 µL/mL, 0.3125 µL/mL, and 0.1562 µL/mL of the prepared extract, respectively. For the HepG2 cell line, the cell viability ratio was calculated as 43.09%, 55.05%, 55.63%, and 61.86% at 1.25 µL/mL, 0.625 µL/mL, 0.3125 µL/mL, and 0.1562 µL/mL of extract, respectively.

Figure 2. Antiproliferative activity of ethanolic extract of *Spartium junceum* L. Flowers.



3.2. Results of HPLC Analysis

HPLC analysis of the ethanolic extract of SJ flowers shows the presence of chlorogenic, cinnamic, ferulic, *p*-coumaric, and protocatechuic acids, epicatechin, and luteolin. Ferulic acid had the highest concentration (2583.3 µg/mL) followed by chlorogenic acid with a concentration of 571.2 µg/mL, then *p*-coumaric acid with a concentration of 545.6 µg/mL, with the presence of epicatechin, luteolin, cinnamic acid, and protocatechuic acid with concentrations of 280.1, 159.4, 34.7 and 12.9 µg/mL, respectively (Table 3). The chemical structures of determined compounds in the ethanolic extract are shown in Figure 3. The open structures of these molecules were drawn with the ChemDraw Professional software program.

Figure 3. Chemical structures of determined compounds in the ethanolic extract of SJ flowers.

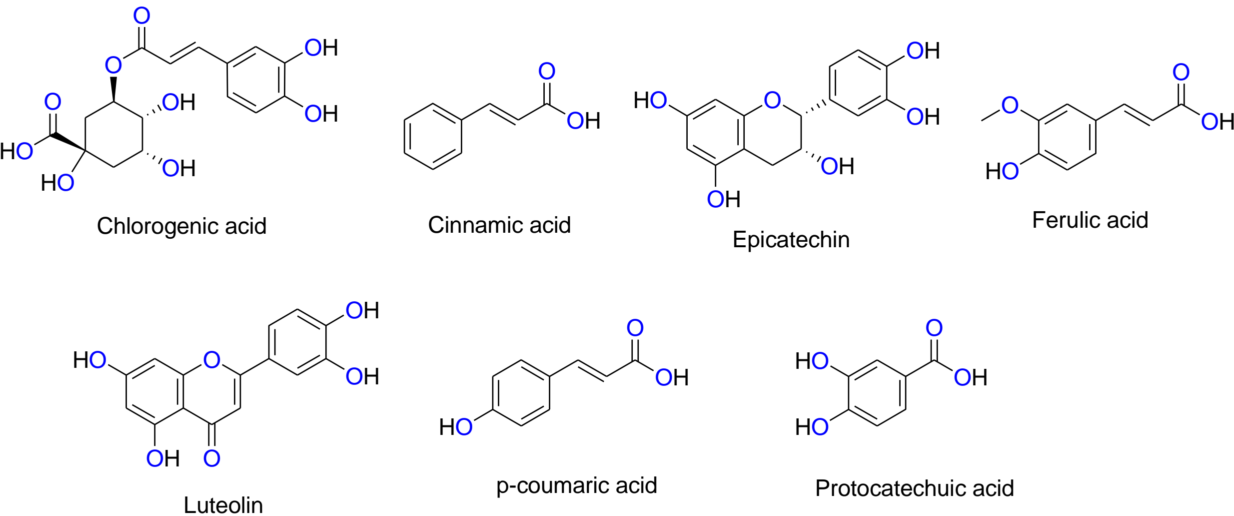


Table 3. Concentrations of the main phenolic compounds identified in the ethanolic extract of SJ flowers.

Phytochemicals	Concentrations (µg/mL)
Chlorogenic acid	571.2
Cinnamic acid	34.7
Epicatechin	280.1
Ferulic acid	2583.3
Luteolin	159.4
p-coumaric acid	545.6
Protocatechuic acid	12.9

4. DISCUSSION

SJ, also known as "Spanish broom", is a perennial erect shrub widespread in the Mediterranean. There are many studies proving that the flowers of this plant have anti-ulcerogenic, antitumor, analgesic, anti-inflammatory, antiviral, and antioxidant properties (Yeşilada *et al*., 2000b; Nanni *et al*., 2018; Menghini *et al*., 2006; Duman *et al*., 2019). However, when the literature is reviewed, it is seen that only a few studies show the anticancer activity of flowers (Abusamra *et al*., 2015; Cerchiara *et al*., 2012). In the previous studies, the anticancer activity of flowers was investigated on different cell lines. Abusamra *et al*. tested the cytotoxic effect of crude hydromethanolic extract prepared from SJ flowers towards the glioblastoma tumor cell line (U-373) (Abusamra *et al*., 2015). They found the IC_{50} value as 1602 µg/mL. So, the hydromethanolic extract of SJ flowers appeared to have weak cytotoxic activity. Cerchiara and coworkers screened the antitumor effect of SJ aromatic water against melanoma (RPMI 7932), leukemia (K562), breast (MCF7-Bart and MCF7-ICLC), and colon adenocarcinoma (SW480) cell lines (Cerchiara *et al*., 2012). They found that the SJ aromatic water had an antitumor effect on these cancer cell lines. Furthermore, they also investigated the toxic effect of SJ aromatic water on the healthy human cell line (NCTC 2544). They found that the aromatic water of SJ has selectivity in normal cell lines compared to cancer cell lines. In the present study, the prepared extract of SJ was screened towards MDA-MB-231 and HepG2 cell lines to find out its antiproliferative activity *in vitro*. It was determined that the extract, which was used in ranging from 0.1562 µL/mL to 5 µL/mL, was effective against both cell lines. Particularly, the extract was found to be more effective in the liver cancer cell line than the breast cancer cell line. In other words, in the study conducted by Cerchiara et al., as in our study, it was observed that the extract had a high cytotoxic effect on cancer cell lines (MDA-MB-231, HepG2) (Cerchiara *et al*., 2012).

Phytochemical analysis of plant extract revealed the presence of several phenolic compounds such as cinnamic acid and its derivatives (chlorogenic, *p*-coumaric, and ferulic acids), protocatechuic acid (3,4-dihydroxybenzoic acid), epicatechin and flavone luteolin. In the literature, there are many studies in which the antiproliferative effects of various plant extracts are attributed to the phenolic compounds in the phytochemical composition of the plants. For example, a study conducted by Vale et al. proved the antiproliferative and antimetastatic potential of cinnamic acid derivatives on melanoma (Vale *et al*., 2022). The antiproliferative effects of luteolin, *p*-coumaric acid, and protocatechuic acid against MCF-7 human breast cancer cell lines have been reported (Zheng *et al*., 2017). Some studies in the literature have shown that epicatechin-rich extracts have *in vitro* antiproliferative effects at high doses (Horie *et al*., 2005; Philips *et al*., 2009; Singh *et al*., 2011). *Ficus carica* L. latex was found to have antiproliferative activity toward numerous cell lines and Ultra Performance Liquid Chromatography coupled with mass spectrometry (UPLC-MS) analysis revealed that various

phenolic secondary metabolites could be responsible for this activity (Yahiaoui *et al*., 2022). A study conducted to analyze the change in the antiproliferative effect of Rhodiola after *in vitro* digestion revealed that gastrointestinal digestion significantly reduced the levels of total phenol and flavonoid content and antiproliferative activity potential of the extract (Zhang *et al*., 2022). Here, the prepared extract was tested in breast and liver cancer cell lines. The results show that the SJ extract had high antiproliferative activity on screened cell lines for 48 h incubation times.

5. CONCLUSION

In conclusion, this study indicated that SJ flower extract could inhibit proliferation in the selected cell lines, possibly due to its rich phenolic phytochemical profile, and the obtained findings from the current study led us to believe that SJ could be a promising antiproliferative agent candidate.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Fatma Tuğçe Gürağaç Dereli: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Senem Akkoç**: Investigation, Methodology, Validation, and Writing.

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Novel carbazole alkaloid from *Murraya koenigii* (l.) Spreng

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Abstract: Background: The plant *Murraya koenigii* (L.) Spreng.native to Pakistan and India and a part of the Rutaceae family, plays a vital role in the Indian Ayurvedic medicine system. Studies have shown that this plant's bark, roots, and leaves contain many carbazole alkaloids. These alkaloids are known to have substantial therapeutic properties.

Methods:
The crushed and powdered organs of *M. koenigii* were extracted with several solvents (acetone, chloroform, and methanol). Then, using column chromatography, a dull brown oily substance, MK- 1 (1.05 g), was obtained with chloroform and methanol (7:3).

Result
We have successfully isolated a new carbazole alkaloid, 3-geranyl 8-hydroxy 6, 7-di methoxy 3’, 3’-dimethyl 1, 2-pyranocarbazole, from the seeds of *M. koenigii*. The structure was further elucidated by cross-referencing our NMR, UV, IR, and MS data with that found in the published literature.

Conclusion:
Comparing the previously published literature data clearly explained that this alkaloid is new to *M. koenigii*.

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Rutaceae,
Carbazole alkaloid,
Pyranocarbazole.

1. INTRODUCTION

A significant breakthrough of the current time will be identifying various rejuvenating molecules that may halt or minimize the pathology of multiple illnesses. The potential for adverse effects and health concerns associated with synthetic chemicals has prompted the quest for natural compounds to replace them. Several phytoconstituents have offered superior therapeutic benefits to standard medical therapy.

Faisalabad is geographically situated amidst the Chenab River and the Ravi River, two prominent water bodies in Pakistan. The Chenab River exhibits a westward flow, whereas the Ravi River is situated towards the southeast. According to data on medicinal plants, this region

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has many plant species. Various medicinally important "Rutaceae" species have a wide distribution. Some of the plants in this family, which have great medicinal value, also are found in Faisalabad. Phytochemical investigations revealed that medicinal plants in the Rutaceae family produce many active chemicals responsible for disease-curing activities. As a result of some highly active compounds, medicinal plants of the Rutaceae family have proven to be more helpful. *Murraya koenigii* (L.) Spreng. (Family Rutaceae) is indigenous to India, Pakistan, Azerbaijan, and numerous Asian nations (Chopra *et al*., 2002; Satyavati *et al*., 1987). In India and Pakistan, *Murraya koenigii* is known as *kathnim, mitha neem, curry patta, gandhela, barsanga*. People have used the organs of *Murraya koenigii* for a long time to make herbal remedies like the leaves to help ease morning sickness; they can also treat diarrhoea and sniffles. Moreover, *Murraya koenigii* branches are often used to clean teeth and build teeth and gums (Chopra *et al*., 2002; Satyavati *et al*., 1987; Gautam *et al*., 2020; Aniqa *et al*., 2021; Batool *et al*., 2020).

The essential oils extracted from this plant have anti-inflammatory, analgesic, and anti-amoebic properties and are used to treat various ailments. The astringent and stimulating properties of the leaves and other organs are employed to treat cuts, joint discomfort, and other bodily aches, as well as anticancer. This plant has identified many phytochemicals, mainly carbazole alkaloids (Abeysinghe *et al*., 2021; Balakrishnan *et al*., 2020). The biologically active carbazole alkaloids in *Murraya koenigii* make this plant a go-to for medicinal purposes, such as cytotoxic, anti-microbial, anti-bacterial, anti-tumour, antioxidant, hypoglycaemic, anti-inflammatory and analgesic also used to treat leprosy (Abeysinghe *et al*., 2021; Balakrishnan *et al*., 2020; Mandal, 2016; Tripathi *et al*., 2018; Knölker & Reddy, 2008). Therefore, we have chosen *Murraya koenigii*, for our research in light of the above context, abundantly found in Faisalabad, Pakistan.

2. MATERIAL and METHODS

2.1. Instrumental

UV spectrometer Perkin-Elmer Lambda Bio 20 (Perkin Elmer, USA) was used to record the ultraviolet absorption spectrum. On Perkin-Elmer 1710 Fourier transform spectrometer (Perkin Elmer, USA), IR spectroscopy was carried out utilising the KBr disc. As an internal standard, tetramethylsilane (TMS) is used to calculate δ values (ppm). The FEBMS were recorded using the JEOLSX 1021/DA-6000 mass spectrometer (JEOL Ltd. Japan). The Bruker AVANCE DRX-400 (German) was used to record NMR spectra (400, 100 MHz). Silica gel (60–120 mesh) was used for column chromatography. The chemicals and reagents used in this experiment were AR quality from E-Merck (Pakistan).

2.2 Plant material

Murraya koenigii seeds were gathered in June 2022 throughout remote rural areas of Faisalabad District, Punjab province. Faisalabad is a city situated between 31°25′0″N latitude and 73°5′28″E longitude. The collected plant specimens were shown to be authentic by the botany department of Government College University in Faisalabad, Pakistan.

2.3 Extraction

It is best to utilise it as soon as it is collected and dried because dried material that has been stored for an extended period might alter dramatically. *Murraya koenigii* air-dried seeds (1.2 kg), were crushed into a fine powder and defatted with petrol-ether (3 Lx5 times). Subsequently, the sample was subjected to Soxhlet extraction using acetone, chloroform, and methanol. We only considered the chloroform extract for further investigation. A rotator was used to evaporate the chloroform extract below 50 ^{0}C temperature to produce a reddish-brown substance (28.6 g). The chloroform extract was reddish-brown (28.6 g) after being evaporated in a rotator at

temperatures below 50 ^{0}C. Next, column chromatography was performed on this brownish-red material. After being well mixed, a silica gel solution, 135 g in pet.-ether, was put into a 150 mm long column with a circular area crosssection 50 mm in diameter.

After the absorbent had finished settling, the column was opened so the extra pet.-ether could flow through it. The column was finally settled after the slurry was digested to produce a methanolic extract using silica gel in a pet-ether. Increasingly polar solvents and solvent combinations were used to elute the column without interruption. A dull brown oily substance **MK-1** (1.05 g) was obtained with chloroform and methanol (7:3). Likewise, the alcoholic extract was eluted in the column with increasing polarity solvents and solvent mixtures. Elution with acetone: methanol (7:1) afforded **MK-2** (0.57 g). Similarly, after being eluted with a mixture of chloroform and ethyl acetate (6:4), the ethyl acetate extract produced the chemical **MK-3** (0.35 g).

Compound MK-1: brown oily substance, $C_{40}H_{40}N_2O_6$; UV (MeOH) λ_{max} (logε) nm: 211.0 (4.60), 240.6 (4.66), 285.4 (4.40 sh), 295.2 (4.56), 325.2 (3.90), 342.2 (3.92), and 356.6 (3.86); IR (KBr) ν_{max} : 3390, 2934, 2875, 1671, 1468, 1459 (*gem* dimethyl), 1102, 1076, 999 and 907 cm^{-1}; ^{1}H NMR(400 MHz, $CDCl_3$) δ: 10.69 (1H, brs, -OH), 9.30(1H, brs, -NH), 7.91(1H, brs, H-5),7.66(1H, brs, H-4), 5.68 (1H, d, *J* = 9.5 Hz, H-1′),5.59 (1H, d, *J* = 9.5 Hz, H-2′), 5.16 (1H, d, *J* = 7.1 Hz, H-6″), 5.09 (2H, d, J= 7.1 Hz, H-2″), 3.35 (2H, d, J= 7.2 Hz, H-1″), 2.09 (2H, d, J= 7.5 Hz, H-5″), 1.98 (2H, d, J= 7.5 Hz, H-4″), 1.47 (3H, s, 10″ -CH_3), 1.54 (3H, s, 8″ -CH_3), 1.45 (3H, s, 9″ -CH_3), 1.37(3H, s, 3′ -Me), 1.29 (3H, s, 4′ -Me), 3.96 and 4.02 (6H, s, 8, 9 -OCH_3); ^{13}C NMR(100 MHz, $CDCl_3$) δ: 153.4(C-2), 151.4(C-6), 136.2 (C-3″), 134.9 (C-9a), 132.9(C-7″), 128.7 (C-8a), 106.2 (C-1), 138.1 (C-8), 127.8 (C-2′), 136.2(C-7), 125.3 (C-6″), 123.1 (C-2″), 123.9 (C-4), 121.2 (C-4a), 117.4 (C-1′), 125.3 (C-3), 122.6(C-4b), 98.7(C-5), 77.1(C-3′), 59.1(-OCH_3), 57.2 (-OCH_3), 38.6 (C-4″), 29.8 (C-1″), 28.7 (5′ -CH_3), 28.4(4′ -CH_3), 27.1 (C-5″), 23.7(9″ -CH_3), 19.1(8″ -CH_3), 17.5(10″ -CH_3); Mass spectra m/z: 461[M] $^+$, 324[M-$C_{10}H_{17}$] $^+$, 137

3. RESULTS

The compound was obtained as a brown oily substance. According to the EIMS study of this compound, a quasimolecular ion *m/z* 462 $[M+H]^+$ was found, compatible with a molecular weight of 461 and molecular formula $C_{29}H_{35}NO_4$. This compound also produces blue-violet colour spots with H_2SO_4 (conc.), characteristic of the carbazole alkaloids (Chakraborty & Roy, 2003; Chakraborty & Roy, 1991). Ultraviolet spectrum of this compound exhibited typical absorptions λ_{max} at 211.0 (4.60), 240.6 (4.66), 285.4 (4.40 sh), 295.2 (4.56), 325.2 (3.90), 342.2 (3.92), 356.6 (3.86) characteristic of a carbazole skeleton (Chakraborty & Roy, 2003; Chakraborty & Roy, 1991). Infrared spectra of this chemical revealed absorption bands at 3390, 2934, 2875, 1671, 1468, 1459, 1102, 1076, 999, and 907 cm^{-1}, which revealed a pyrano carbazole system. According to the spectroscopic evidence, the compound was carbazole and had a pyran ring, attached to the carbazole at C-1 and C-2 (Chakraborty & Roy, 2003; Chakraborty & Roy, 1991).

A broad singlet observed at δ 9.30 in the ^{1}H NMR spectrum corresponds to the –NH proton, while an additional singlet observed at δ 10.69 is assignable to the –OH proton. Other two singlets have been observed at δ 7.66 and 7.91 for carbazole ring, H-4 and H-5 protons. The singlets nature of H-4, and H-5 confirmed that the remaining positions of carbazole were substituted. Signals were observed at δ 3.35 (2H, d, *J*= 7.1 Hz, H-1″), 1.98 (2H, d, *J* =7.5 Hz, H-4″), and 2.09 (2H, d, *J*= 7.5 Hz, H-5″), allocated for methylene protons. Furthermore, signals detected at δ_H 5.09 (1H, d, *J* = 7.1 Hz, H-2″), and 5.16 (1H, d, *J*= 7.1 MHz, H-6″), were attributed to two methine protons (Kapil, 1971; Chakraborty, 1977; Bhattacharyya & Chakraborty, 1987).

Additionally, singlets have been found in ^{1}H NMR at δ 1.47 (3H, s, 8″ -CH_3), 1.45 (3H, s, 9″ -CH_3), and 1.54 (3H, s, 10″ -CH_3) were unambiguously assigned to three methyl groups. Based on these findings, the molecule has a geranyl-substituted group. The ^{13}C chemical shift of δ 115.3 for the geranyl-substituted carbon strongly suggests the presence of the geranyl group in C-3. In HMBC correlations of H-1″ at δ 3.35 with C-3 (δ 115.3), C-2 (δ 153.4), and C-4 (δ 122.1), the geranyl moiety was confirmed to be located at C-3 (Kapil, 1971; Chakraborty, 1977; Bhattacharyya & Chakraborty, 1987). Furthermore, doublets are at δ 5.68 (1H, d, 9.5 Hz, H-1'), and 5.59 (1H, d, 9.5 Hz, H-2') are allocated to double bonds protons H-1' and H-2' of the pyran ring. Additionally, spectra revealed two peaks at δ_H 1.37 and 1.29 associated with gem-dimethyl groups on pyran rings (Kapil, 1971; Chakraborty, 1977; Bhattacharyya & Chakraborty, 1987).

In addition, two singlets were seen at δ 3.96 and 4.02 (6H, s, 2-OCH_3) associated with two methoxy groups in the carbazole structure at C-6 and C-7. Furthermore, signals observed at δ_H 4.02 / δ_C 56.3 and δ_H 3.96 / δ_C 55.8 were assignable for methoxy protons positioned at C-6 and C-7. In the mass spectra, the existence of a fragment with a mass of *m/z* 137 indicated the existence of a geranyl group in the structure (Scheme 1). Accordingly, based on these spectral shreds of evidence and literature comparisons, the isolated compound should be 3-geranyl 8-hydroxy 6, 7-di methoxy 3', 3'-di methyl 1, 2-pyranocarbazole (**MK-1**). This compound is isolated for the first time from the seeds of *Murraya koenigii*.

The two known compounds were also isolated and identified as 3,5-dimethyl-3-(4-methylpent-3-enyl)-11*H*-pyrano[3,2-a]carbazole (**MK-2**, mahanimbine), 3,3,5-trimethyl-11H-pyrano[3,2-a]carbazole (**MK-3**, Girinimbine) through matching their NMR values to one previously published (Kapil, 1971; Chakraborty, 1977; Bhattacharyya & Chakraborty, 1987).

Mahanimbine lower blood glucose, also prevents obesity, mitigates metabolic abnormalities caused by excessive fat consumption, and has anti-aging and anti-anxiety effects. It fights against leukemia also bladder, and pancreatic cancer (Hobani, 2022). Whereas girinimbine has been shown to have anticancer action in vitro, and it also has potent antioxidant and gastro-protective properties (Singh, et. al., 2023).

Scheme 1. (MK-1)

MK-2 **MK-3**

3-geranyl 8-hydroxy 6, 7-di methoxy 3', 3'-di methyl 1, 2-pyranocarbazole (**MK-1**)

m/z: 461.26 (100.0%)

324.12

137.13

4. DISCUSSION and CONCLUSION

Accordingly, based on these spectral shreds of evidence and literature comparisons, the isolated compound should be called as 3-geranyl 8-hydroxy 6, 7-di methoxy 3', 3'-dimethyl 1, 2-pyranocarbazole. This compound is isolated for the first time from the seeds of *Murraya koenigii*.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Arifa Mehreen, Shagufta Kamal: Methodology, Supervision, and Validation. **Sevinj Musayeva Vagif:** Resources, Visualization. **Muhammad Qaisar:** Software, Formal Analysis. **Sumia Urainab:** Writing original draft. **Asad Ullah**: Investigation.

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

In-vitro anti-diabetic, anti-Alzheimer, anti-tyrosinase, antioxidant activities of selected coumarin and dihydroisocoumarin derivatives

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Abstract: Benzo-α-pyrone structured coumarin derivatives are secondary metabolites first obtained from *Coumarouna odorata* in 1822. Coumarin and its structural isomer dihydroisocoumarin derivatives are found in many different sources in nature. Several different bioactivities of these compounds have been reported. In this study, preliminary activity screening and comparison of four purchased coumarin derivatives (esculetin, esculin monohydrate, umbelliferon, scoparone) and four previously isolated 3-phenyl-3,4-dihydroisocoumarin derivatives (thunberginol C, scorzocreticoside I, scorzocreticoside II, scorzopygmaecoside) from a medicinal plant were carried out by *in-vitro* methods. α-Glucosidase, acetylcholinesterase, butyrylcholinesterase, tyrosinase inhibitor activities and antioxidant potentials of the compounds were evaluated. Consequently, thunberginol C (free – not glycosylated form of 3,4-dihydroisocoumarin structure) showed better potential in all enzyme inhibitory activities compared to coumarin structure. Particularly, α-glucosidase inhibitory activity of this compound with a very low IC_{50} value (94.76±2.98 µM) compared to standard acarbose (1036.2±2.70 µM) should be noted. Glycosylation and/or methoxy substitution of 3,4-dihydroisocoumarin structure resulted a significant decrease in all tested enzyme inhibitory activities. The structures of esculin MH, umbelliferone, scoparone, scorzocreticoside I, and scorzopygmaeceoside might be considered in further synthetic studies as selective acetylcholinesterase inhibitors. Thunberginol C has a promising potential in tyrosinase inhibitory activity. Esculetin and thunberginol C showed the best results with high potentials in antioxidant activity via 2,2-diphenyl-1-picryl-hydrazyl-hydrate free radical scavenging, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid cation radical decolorization, and cupric ion reducing antioxidant capacity assays compared to the standards.

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1. INTRODUCTION

The use of medicinal plants is thought to be as old as first humans. The isolation of pure active compounds from these plants and turning them into drugs dates back to the 19th century. To date, many different secondary metabolites have been identified and presented to the usage and research in the pharmaceutical industry. The first of the coumarin derivatives, one of the important secondary metabolite groups, was isolated from tonka beans (*Dipteryx odorata* (Aubl.) Willd.) in 1822. Coumarin compounds, which are essentially in the benzo-α-pyrone

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structure, are found both in glycoside and free form in many different plants. Coumarin derivatives are compounds with different characteristic odors as in freshly mown grass. The reported pharmacological activities of coumarin derivatives have a broad spectrum. Examples of these are anti-microbial, anti-HIV, anticancer, MAO inhibitory, anti-diabetic, anti-inflammatory, anti-parasitic, antihypertensive, anti-Alzheimer, anti-convulsant, and antioxidant activities (Bruneton, 1995; Evans & Evans, 2009; Anand *et al*., 2012; Venkata Sairam *et al*., 2016; Srikrishna *et al*., 2018).

The new compounds that are formed as a result of the interchange of the oxygen atom and the keto group in the α-pyrone ring of the coumarin compounds are called isocoumarins. Isocoumarins and their 3,4-dihydro derivatives are natural compounds like coumarins and have been isolated from different sources such as plants, microbial strains, venoms, insects, and marine organisms. The biological activities of isocoumarins, which is a smaller group than coumarins, may show more diversity. The presence of both sweetening (phyllodulcin) and bitter (mellein) isocoumarins with the same main structure may be proof of this. Some of the reported pharmacological properties of isocoumarins are anti-microbial (anti-biotic, anti-malarial, anti-fungal), hepatoprotective, gastroprotective, neuroprotective, anti-inflammatory, anti-diabetic, sweetening, anti-allergic, and immunomodulatory activities. Among the 3,4-dihydroisocoumarins, 3-phenyl substituted derivatives have drawn attention with their prevalence in nature and similar pharmacological properties including above-mentioned activities (Braca *et al*., 2012; Saeed, 2016; Saddiqa *et al*., 2017; Çiçek *et al*., 2018).

There are many molecules from these groups that have become natural, semi-synthetic, synthetic drugs or drug candidates (Kontogiorgis *et al*., 2012). However, more studies are needed for these derivatives to be used more in the pharmaceutical industry. It is particularly important to screen new natural derivatives for related activities and to compare these pharmacologically important scaffolds. This study aims to investigate the inhibitory potential against several enzymes (α-glucosidase, AChE, BChE, tyrosinase) and antioxidant activity of selected four coumarin derivatives (esculetin, esculin monohydrate, umbelliferon, and scoparone) and four previously isolated 3-phenyl-3,4-dihydroisocoumarin derivatives (thunberginol C, scorzocreticoside I, scorzocreticoside II, and scorzopygmaecoside) from a medicinal plant considering the reported pharmacological properties of the involved structures.

2. MATERIAL and METHODS

2.1. Chemicals and Compounds

PNPG (p-nitrophenol, α-D-glycopyranoside), enzyme α-glucosidase type I (E.C. 3.2.20), disodium hydrogen phosphate, sodium azide, sodium dihydrogen phosphate, acarbose, DMSO, AChE (acetylcholinesterase), BChE (butyrylcholinesterase), and tyrosinase from mushroom (E.C. 1.14.18.1) were obtained from Sigma–Aldrich/Merck. Acetylthiocholine iodide was purchased from Applichem and butyrylthiocholine iodide was Fluka branded. All other chemicals were of analytical grade.

Esculetin, esculin monohydrate, umbelliferone, and scoparone were purchased from Sigma-Aldrich. Thunberginol C, scorzocreticoside I, scorzocreticoside II, and scorzopygmaecoside were isolated and identified previously (Şahin *et al*. 2020a; Şahin *et al*. 2020b).

2.2. Anti-Diabetic Activity

α-Glucosidase inhibitory activity was employed for determination of anti-diabetic potential of the compounds (Trinh *et al*., 2016). Na_2HPO_4, NaH_2PO_4 and ultra-pure water were used to prepare a buffer with pH 7.5 containing NaN_3 (0.02 %). In brief, 100 µL of the compounds dissolved in buffer with 10 % DMSO (8 concentrations between 800 – 6.25 µM), and 80 µL of enzyme solution were added to all wells. After incubation (28 °C, 10 min) 20 µL of PNPG

(substrate) was added. The blank wells contained enzyme, substrate, and buffer with 10 % DMSO. No background well was used since using slopes instead of absorbance eliminates the potential absorbances due to the color of the compounds. Absorbance measurement at 405 nm every 40 s for 35 min and incubations were performed with a microplate photometer BioTek Power Wave XS branded. An oral inhibitor, acarbose, was employed as control. α-Glucosidase inhibition % was calculated using the following formula:

$$\text{Inhibition \%} = (\text{Slope}_{blank} - \text{Slope}_{sample}) / \text{Slope}_{blank} \times 100$$

2.3. Anti-Alzheimer Activity

A colorimetric method developed by Ellman *et al*. was used with minor changes to evaluate AChE and BChE inhibitory activities of the compounds (Ellman *et al*., 1961; Yıldız *et al*., 2022). Each well finally contained 150 µL of buffer, 10 µL of compound solutions, 20 µL of enzyme solution (BChE or AChE), 10 µL of DTNB [5,5-dithiobis (2-nitro benzoic acid)], and 10 µL of the either acetylthiocholine iodide or butyrylthiocholine iodide. The incubation times at 25 °C were 15 minutes before DTNB was added and 10 minutes after iodides were added. Above-mentioned plate reader was used for incubations and measuring the absorbances at 412 nm. Galantamine was used as positive control and sample solvent was used as blank. % inhibitions were calculated according to following equation.

$$\text{Inhibition \%} = (A_{blank} - A_{sample}) / A_{blank} \times 100 \text{ (A: Absorbance)}$$

2.4. Anti-Tyrosinase Activity

Another colorimetric method was used to determine the anti-tyrosinase potentials of the compounds (Hearing & Jiménez, 1987). L-DOPA (0.5 mM) was the substrate for tyrosinase enzyme. A phosphate buffer with pH 6.8 was used. Compounds were prepared in a series of concentration and a pre-incubated with enzyme solution for 10 minutes at room temperature. The substrate was added to start the enzymatic reaction. 20 minutes incubation was carried out and the absorbance was measured at 475 nm at 37 °C. Kojic acid was employed as control. Same equation given in anti-Alzheimer activity section was used to calculate the percentage of the inhibitory activity of the samples.

2.5. Antioxidant Activity

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) cation radical decolorization, and cupric ion reducing antioxidant capacity (CUPRAC) assays were preferred to evaluate the antioxidant potentials of the compounds. All assays were carried out according to previously published literature (Blois, 1958; Re *et al*., 1999; Apak *et al*., 2004; Yıldız *et al*., 2022).

All assays were performed in triplicates (Student's t-test $p<0.05$) and the results were reported as mean ± SD. IC_{50} calculations were performed with GraphPad Prism 8.0.1.

3. RESULTS and DISCUSSION

Enzyme inhibitory and antioxidant activities of selected coumarins and 3-phenyl-3,4-dihydroisocoumarins are presented in Table 1 and Table 2 respectively.

Enzyme inhibition is one of the most studied modes of action in the discovery of new drug molecules. It is therefore not surprising that many inhibitors of different enzymes are found in clinical use. Glucosidase inhibitors prevent α-glucosidase from hydrolyzing oligosaccharides to monosaccharides in human intestine. Thus, they contribute to the treatment/care of Diabetes mellitus (DM) patients by preventing postprandial hyperglycemia. Keeping the blood glucose level under control in these patients is very crucial, particularly for preventing/delaying of chronic complications of DM such as retinopathy and neuropathy (Maurya *et al*., 2020).

Selected coumarins; esculetin, esculin MH, umbelliferone, scoparone and dihydroisocoumarins; thunberginol C, scorzocreticoside I, scorzocreticoside II, scorzopygmaeacoside (Figure 1) were tested against this enzyme *in-vitro*. IC_{50} values of all tested compounds were above 800 μM except esculetin and thunberginol C (Table 1). These compounds showed higher potency than the standard acarbose which is a clinically used oral inhibitor of the enzyme. It should be noted that IC_{50} value of thunberginol C is approximately ten times lower than that of acarbose. Furthermore, the results suggest that glycosylation and/or methoxy substitution instead of free hydroxyl in both coumarins and dihydroisocoumarins decrease the activity significantly. Considering the potencies of esculetin, umbelliferon, and scoparone together, it can be deduced that at least two free hydroxyl substitution is crucial for higher activity. In addition to that, the same pattern in dihydroisocoumarins suggests that meta positioning of phenolic hydroxyl groups may result higher activity than that of ortho positioning. This is the first report on α-glucosidase inhibitory effect of the tested compounds except esculetin, umbelliferone and scoparone. Other data about these three compounds are in accordance with the previous literature (Nurul Islam *et al*., 2013; Karakaya *et al*., 2018).

Table 1. Enzyme inhibitory activity results of the compounds.

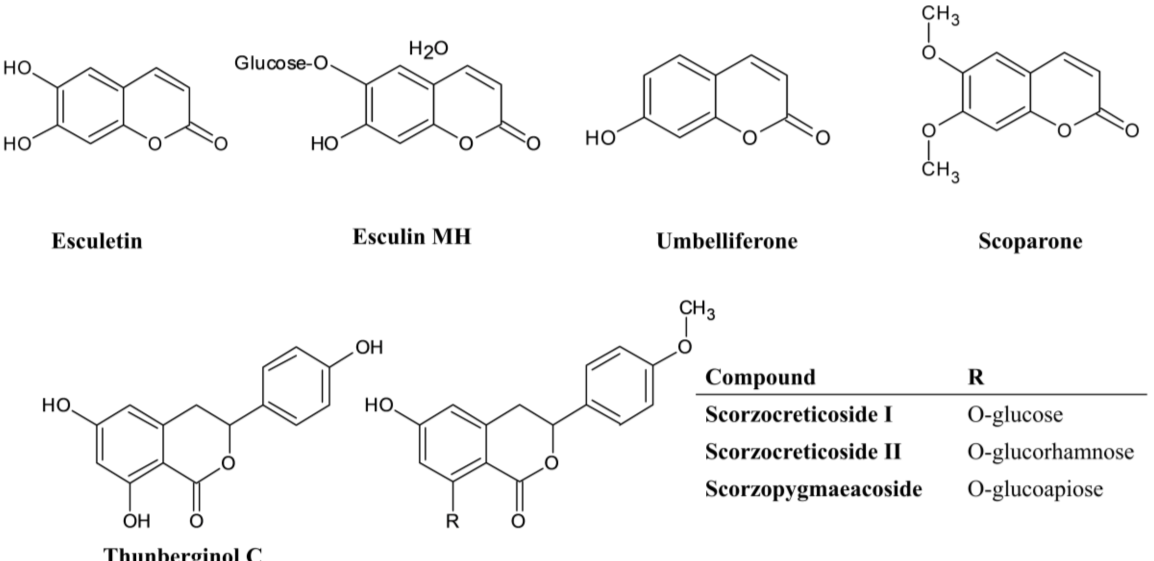
	IC_{50} (μM)			
	α-glucosidase	AChE	BChE	Tyrosinase
Esculetin	374.00 ± 0.70	135.53 ± 1.59	196.99 ± 0.36	NT
Esculin MH	>800	118.82 ± 2.20	>1000	>1000
Umbelliferone	>800	209.61 ± 0.74	>1000	NT
Scoparone	>800	236.96 ± 1.41	>1000	>1000
Thunberginol C	94.76 ± 2.98	82.41 ± 1.30	137.25 ± 1.01	90.25 ± 1.67
Scorzocreticoside I	>800	133.90 ± 0.43	>1000	531.16 ± 3.27
Scorzocreticoside II	>800	265.78 ± 0.42	340.20 ± 0.36	>1000
Scorzopygmaeacoside	>800	261.03 ± 1.38	>1000	240.91 ± 1.65
Acarbose[a]	1036.2 ± 2.70	NT	NT	NT
Galanthamine[b]	NT	5.78 ± 0.02	16.58 ± 0.18	NT
Kojic acid[c]	NT	NT	NT	15.72 ± 0.14

Values are means of three parallel measurements ± Standard deviation.
a Standard compound for α-glucosidase
b Standard compound for AChE and BChE
c Standard compound for Tyrosinase
NT: Not tested.

One of the approaches in the treatment of neurodegenerative Alzheimer's disease characterized by cholinergic abnormalities is the inhibition of AChE and BChE enzymes (Francis *et al*., 2010). All tested compounds showed low-moderate inhibitory activities on these enzymes (Table 1). Esculin MH, umbelliferone, scoparone, scorzocreticoside I and scorzopygmaeacoside were more selective towards AChE, while esculetin, thunberginol C and scorzocreticoside II acted as dual inhibitors. The most potent AChE inhibitor was thunberginol C. The glycosylation and/or methoxy substitution instead of free hydroxyl of this compound led to lower potentials just as in case of α-glucosidase inhibition. However, glycosylation resulted in higher selectivity towards AChE enzyme considering potentials of scorzocreticoside I and scorzopygmaeacoside. Similarly, glycosylation, methoxy substitution instead of free hydroxyl, and loss of a free hydroxyl group led to the same selectivity in coumarin derivatives. Current literature provides studies conducted on AChE/BChE inhibitory activities of umbelliferone, esculetin, scoparone and thunberginol C with different results. However, most of the studies report moderate-high potencies (Adhami *et al*., 2014; Ali *et al*., 2016; Hwang *et*

al., 2021). Although, none of the compounds showed comparable potential with positive standard galantamine, results may contribute to the field by being new models for selective synthetic inhibitors. Despite the decrease in AChE activity in advanced stages of Alzheimer's disease, the increase in BChE activity shows that selective inhibitors may be more useful in the treatment (Lane *et al*., 2006).

Figure 1. Structures of selected coumarin and dihydroisocoumarin derivatives.



Tyrosinase is a multifunctional oxidase which mediates producing melanin from tyrosine. Thus, its inhibition has different potentials in skin-whitening, skin cancer, neurodegeneration, and undesired browning of foods (Bonesi *et al*., 2019). Selected coumarin derivatives revealed no activity against tyrosinase enzyme at tested concentrations (Table 1). Esculetin and umbelliferone were not tested against this enzyme since several studies reported these compounds as substrates for the enzyme (Munoz-Munoz *et al*., 2007; Garcia-Molina *et al*., 2013). No previous study has been found on the anti-tyrosinase activity of scoparone. However, low inhibitory activity result of esculin MH is in accordance with the previous literature (Masamoto *et al*., 2003). The most potent inhibitor against tyrosinase enzyme among the tested compounds was thunberginol C with a potential approximately 6 times weaker than the positive control kojic acid. The negative impact of the glycosylation and/or methoxy substitution of tested dihydroisocoumarins on the inhibitory activity was valid for this enzyme too.

Oxidative stress in humans caused by several reasons such as stress, unhealthy diet, chemicals etc., is associated with many diseases. Thus, antioxidants which can keep the oxidative stress in desired limits are suggested to decrease the risk of them. Main antioxidant sources of humans are natural phytochemicals provided by traditional medicinal and edible plants (Sen & Chakraborty, 2011). In this context, antioxidant activities of the selected compounds were evaluated and esculetin appeared to be the most potent antioxidant in every tested method with better potencies than used standards (Table 2). Furthermore, a significant decrease was determined in case of glycosylation, methoxy substitution instead of free hydroxyl, and loss of a free hydroxyl group in coumarin derivatives. This structure-activity relationship is valid for dihydroisocoumarin derivatives except scorzocreticoside II in DPPH method. Thunberginol C was determined as a promising antioxidant among the tested dihydroisocoumarins with high potencies comparable to that of the standards. Results are in accordance with previous studies reporting moderate to high antioxidant potentials of coumarin

and dihydroisocoumarin derivatives (Zidorn *et al*., 2005; Wu *et al*., 2007; Witaicenis *et al*., 2014; Mazimba, 2017).

Table 2. Antioxidant activity results of the compounds.

	EC_{50} (μM)		$A_{0.5}$ (μM)
	DPPH Free Radical	ABTS Cation Radical	CUPRAC
Esculetin	8.37 ± 0.22	6.29 ± 0.04	10.26 ± 0.19
Esculin MH	255.39 ± 1.21	201.62 ± 1.56	90.06 ± 1.84
Umbelliferone	539.16 ± 1.08	293.58 ± 2.71	556.33 ± 4.42
Scoparone	182.39 ± 2.72	>1000	>1000
Thunberginol C	126.38 ± 0.07	17.69 ± 1.16	62.22 ± 2.66
Scorzocreticoside I	641.03 ± 5.54	520.34 ± 6.18	>1000
Scorzocreticoside II	65.83 ± 1.22	455.31 ± 2.40	>1000
Scorzopygmaeacoside	536.27 ± 4.16	239.25 ± 2.83	298.69 ± 2.43
BHA[a]	45.31 ± 1.32	8.53 ± 0.24	25.91 ± 0.50
α-TOC[a]	49.88 ± 0.79	14.31 ± 0.32	38.89 ± 0.87
BHT[a]	270.42 ± 1.52	6.42 ± 0.38	32.74 ± 1.52

Values are means of three parallel measurements ± Standard deviation.
a Standard compounds

4. CONCLUSION

Consequently, thunberginol C (free form of 3,4-dihydroisocoumarin structure) showed better potential in all enzymes inhibitory activities compared to coumarin structure. Particularly, α-glucosidase inhibitory activity of this compound with a very low IC_{50} value compared to standard acarbose should be noted. Further toxicological and *in-vivo* activity studies might be considered on this compound to develop a more potent hypoglycemic agent. Glycosylation and/or methoxy substitution instead of free hydroxyl of 3,4-dihydroisocoumarin structure resulted in a significant decrease in all tested enzyme inhibitory activities. Esculin MH, umbelliferone, scoparone, scorzocreticoside I, and scorzopygmaeceoside might be considered in further studies as selective AChE inhibitors. Thunberginol C has a promising potential in tyrosinase inhibitory activity. Esculetin and thunberginol C showed the best results with high potentials in antioxidant activity via DPPH free radical, ABTS cation radical scavenging and CUPRAC assays compared to the standards.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

Authorship Contribution Statement

Hasan Şahin: Conceptualizing the study, Conduction of the assays, Interpreting of the results, and Writing the manuscript.

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Effects of Grayanotoxin-III on different cell lines: in vitro ischemia model

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Abstract: Grayanotoxins (GTXs) are natural products and are mostly found in plants of the *Ericaceae* family, especially in the *Rhododendron*. With their ability to bind to voltage-gated sodium channels, they keep these channels constantly active and cause tissue damage. However, despite this feature, the use of *Rhododendron* leaves or its secondary products as an alternative product is especially common in Turkey. This study aims to evaluate the possible dose-related effects of GTX-III in ischemia-induced *in vitro* cell models. Within the scope of the study, an ischemia model was established in two different cell lines (H9c2 and Cos-7) and treated with various concentrations of GTX-III. In this context, cell viability, cytotoxicity, apoptosis and necrosis were examined. In the results of MTT, a significant decrease ($^{+}p < 0.05$) in cell viability was observed in all GTX-III concentrations in H9c2 cells compared to the control, while a significant difference ($^{+}p < 0.05$) was observed in Cos-7 cells, especially at the 24th hour. LDH cytotoxicity was increased in a dose-dependent manner in both cell models. It was concluded that GTX-III caused apoptosis, and reduced cell viability in ischemia models; however, promoted cell proliferation in healthy cells. Based on the literature review, this study is the first to document the cytotoxic properties and apoptotic potential of GTX-III in an *in vitro* cell culture ischemia model. Our findings support the usage of GTX-III, however it should be remembered that the dose needs to be verified before being used medically.

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1. INTRODUCTION

Rhododendron, comprising about 1215 taxa, is a cosmopolitan genus of the *Ericaceae* family (MacKay and Gardiner 2017). These plants are distributed mainly in Asia, with taxa also native to North America, Europe, Southeast Asia and Australia (Qiang *et al*., 2011; MacKay and Gardiner 2017). More than 25 Grayanotoxin (GTX) isoforms are known to be isolated from *Rhododendron* species (Qiang *et al*., 2011). These toxic agents can be found in the leaves, flowers, nectars, or secondary products (such as honey) of the aforementioned plants (Lim *et al*., 2016). Of these agents, GTX-III is the most toxic.

GTXs' cytotoxicity is caused by the blocking of sodium channels in the excitable cell membranes, preventing transmission. As a result of this blockage, the excitable nerve and muscle cells are kept in a state of continuous depolarization (Bilir *et al*., 2018). GTXs' also lead

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to cardiac toxicity by increasing sodium channel permeability and activating the vagus nerve (Choo *et al*., 2008; Poon *et al*., 2008). Diterpenic GTXs prevent Na^+ channel inactivation (Mladěnka *et al*., 2018). In severe GTX-III intoxications, life-threatening cardiac complications such as complete atrio-ventricular block have been reported with an increase in resting sodium permeability as well as activation of voltage-sensitive sodium channels (Durdagi *et al*., 2014). All GTXs can cause different cardiac arrhythmias (Aliyev *et al*., 2009; Okuyan *et al*., 2010). As a result of studies conducted to elucidate this information, it has been shown that the mechanism underlying GTX-induced arrhythmias is the production of activity triggered by release after potentials (Brown *et al*., 1981).

Rhododendron species are used as traditional medicines for the treatment of inflammation, pain, skin ailments, colds, and gastrointestinal ailments (Popescu *et al*., 2013). Additionally, it is believed that *Rhododendron* honey reduces the risk of coronary heart disease and is also a sexual stimulant (Dubey *et al*., 2009). In addition, it is predicted that the misused dose of GTX-III causes oxidative stress in cells, and many diseases can be triggered by the initiation of this process (Incalza *et al*., 2018).

Apoptosis plays an essential role in cardiovascular and renal diseases, increasing oxidative stress, which is the primary cause of mitochondrial dysfunctions, such as the increase of ROS, saturation of antioxidant systems, and exhaustion of ATP. Programmed cell death is caused by mitochondrial dysfunction. There was a correlation between an increase in apoptosis and a decrease in cardiac functions in previous studies (Ritter and Neyses, 2003; Argun *et al*., 2016). Apoptosis and oxidative stress are known to be associated with many cardiac events (Doğanyiğit *et al*., 2020). Likewise, it is known that they are related with diseases of chronic kidney diseases (Small *et al*., 2012).

To the best of our knowledge, despite this strong evidence for anti-hypertensive effects, the effects and doses of GTX-III itself or the new GTX analogues on the heart muscle and renal cells in cardiovascular or renal diseases have not (yet) been fully investigated. This rationale led us to investigate whether GTX-III had potential dose-dependent effects on kidney cell and cardiomyocyte disease models in the present study.

2. MATERIAL and METHODS

2.1. Materials

GTX-III hemi (ethyl acetate) (CAS Number 4678-45-9) was obtained from Sigma Aldrich (Germany). All chemicals and solvents were obtained as cell culture grade. H9c2 and Cos-7 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD).

2.2. Determination of Cell Type

The H9c2 cell line is widely used as a model in reperfusion-ischemia (RI) studies (Kuznetsov *et al*., 2015). The H9c2 cell line (derived from embryonic rat ventricular tissue and lost its pulsing characteristic) was used in this study due to its similarities (membrane morphology, electrophysiological properties) with primary cardiomyocytes in many ways. At the same time, it represents a homogeneous population of heart cells offering numerous experiments to simulate cardiac pathologies. Cos-7 cells most resemble human fibroblast cells and are thus often called Cos-7 monkey kidney fibroblast or Cos-7 fibroblast-like cells (Condreay *et al*., 1999).

2.3. Determination of Concentration of H_2O_2 and GTX-III

We selected the same H_2O_2 concentration used by Xu *et al*. (2016). 600 µM H_2O_2 was shown to regulate cellular stress responses, proliferation, survival, and differentiation within the scope of this study.

0.5 µM,1 µM, and 10 µM of the GTX-III used in cell culture were based on tissue concentration studies of its analogues. Similar concentrations chosen in this study were also used in other cell culture studies made with GTX-III (Brown *et al*., 1981). GTX-III was freshly dissolved in 0.1% DMSO as a stock solution and diluted with DMEM before use.

2.4. *in vitro* Cell Culture Studies and Experimental Groups

H9c2 and Cos-7 cells were grown in DMEM containing 10% FBS and 1% P/S in standard conditions (5% CO_2, 95% air at 37 °C). The medium change of cells was done every 2 to 3 days. After reaching adequate cell numbers, cells were inoculated in 96-well culture plates for 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) test and in 24-well culture plates for Acridine orange/Propidium iodide (AO/PI) double staining (Türck *et al.,* 2022). After 24 h of incubation with and without H_2O_2, the media were removed from all groups. And, GTX-III prepared in three different concentrations (D1:0.5 µM, D2:1 µM, and D3:10 µM) were applied to the cell after both applications. The untreated cells were considered a "control (untreated group)". Cells treated only with H_2O_2 were named as a "treated group". Also, the cells were treated with only 0.1% DMSO which was regarded as a "solvent control group".

2.5. Cell Viability Assay

The cytotoxic effects of GTX-III on H9c2 and Cos-7 cells were determined using the MTT test. Briefly, H9c2 and Cos-7 cells were trypsinized, and $2x10^4$ cells/well were seeded into 96-well plates for each. At the 24th h following the cultivation, 600 µM H_2O_2 was applied for 120 minutes and then H_2O_2 was removed from the plates and GTX-III concentrations were added. GTX-III concentrations were applied directly to the groups that were not treated with H_2O_2. For the MTT test, GTX-III concentrations were removed from the cells after 24, 48, and 72 h incubation. Fresh culture medium containing 10% MTT solution was added to each well and incubated at 37 °C for 4 h. Isopropyl alcohol was added to the medium to dissolve the formazan crystals formed at the end of the incubation and the absorbance at 540 nm was recorded with a microplate reader (µQuantTM, BiotekW Instruments Inc, USA). The assay was repeated in three independent experiments (n=6). Cell viability percentage was calculated by the following equation 1:

$$\%Cellular\ Viability = \frac{Treated\ Group\ Absorbance}{Control\ Group\ Absorbance} x100 \quad (1)$$

The MTT results of the cells incubated with the standard medium (untreated group) were considered to be 100% viable and the viability percentages of the experimental groups were calculated by comparing them with the control group.

2.6. Apoptotic Staining and Scoring of Apoptotic Cells

Cell death occurs in many ways, from cell skeletal damage, cell contraction, plasma membrane condensation, membrane swelling, nuclear condensation, and DNA fragmentation, when cells enter the apoptosis pathway (Abdel *et al*., 2009). To investigate the potential of apoptosis and the type of death of the cells, the ratio of apoptosis was examined with AO/PI double staining. Staining was performed at 24, 48, and 72 h following the incubation. Briefly, cells were washed gently with PBS, after culture mediums were removed. Cells were stained with the equal volume combination of AO and PI for 20 seconds and washed twice with PBS. The stained sample was viewed under fluorescent microscopy (Olympus IX70, Japan). The apoptosis potential of cells was determined by counting according to the following criteria: viable cells; it has an organised structure and a uniform green core (1); early apoptotic cells, which have bright green areas due to the concentration of chromatin in the nucleus (2); late apoptotic cells; it has intense orange chromatin condensation areas (3); necrotic cells have a uniform orange

nucleus (4). Apoptosis potentials in groups were calculated according to the following equations 2 and 3:

$$\%Apoptotic\ Cells\ = \frac{Total\ number\ of\ apoptotic\ cells\ (early\ or\ late)}{Total\ count\ cell} x100 \tag{2}$$

$$\%Necrotic\ Cells\ = \frac{Total\ number\ of\ necrotic\ cells}{Total\ count\ cell} x100 \tag{3}$$

2.7. Lactate Dehydrogenase Assay

The lactate dehydrogenase (LDH) test is a widely used spectrophotometric test to determine cell membrane damage. The damage potential was determined using the LDH cytotoxicity test. LDH levels were determined in H9c2 cells seeded at $5x10^3$ cells/well and incubated for 24 h. Then, cells were treated with and without H_2O_2. Groups for LDH testing were designed as in MTT analysis. After incubation with experimental solutions, all wells were incubated for another 48 and 72 h. 10 µl of culture medium (cell supernatant) was used for the assay. LDH release was calculated by measuring according to the manufacturer's protocol (Thermo Scientific). Briefly, it was measured at an absorbance of 490 nm using a spectrophotometer (µQuantTM, Biotek® Instruments Inc, USA). In addition, the % cytotoxicity was calculated according to the manufacturer's protocols:

$$\%Cytotoxicity\ = \frac{Compound\ treated\ LDH\ activity - Spontaneous\ LDH\ activity}{Maximum\ LDH\ activity - \ Spontaneous\ LDH\ activity} x100 \tag{4}$$

2.8. Statistical Analysis

GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA, USA) was used in statistical analysis. Differences between groups were analysed using analysis of variance (two-way ANOVA). Data were represented as mean ± standard deviation (SD) with a significance level of $p < 0.05$.

3. RESULTS

3.1. MTT Analysis

As shown in Figure 1, 600 µM H_2O_2 and GTX-III reduced H9c2 myocardial cell viability in the concentration range of 0.5 µM (D1), 1 µM (D2), and 10 µM (D3). A significant decrease was observed in all H_2O_2 application times compared to the control. Similarly, a significant decrease was observed in cell viability at 48 and 72 h when D1 concentration was applied from GTX-III groups following H_2O_2 application, while a significant decrease was observed only at 72 h after D2 and D3 concentration groups were applied ($^*p < 0.05$). Therefore, 48 and 72 h were selected for the LDH test based on these results for H9c2 cells.

There was no significant difference between H_2O_2 and dilutions of H_2O_2+GTX-III groups at all incubation times. In addition, significant increases were observed in D1, D2, and D3 of DMEM-GTX-III groups at 24 and 48 h compared to the control group ($^{\#}p < 0.01$). Also, cell viability was increased significantly within the DMEM-GTX-III concentrations at 72 h ($^{+}p < 0.05$) (Figure 1).

In cell viability experiments with the Cos-7 cell line, it was determined that H_2O_2 significantly reduced cell viability in all time periods ($^*p < 0.05$). This result showed that the ischemia pattern was formed in these cells as well. It was observed that different concentrations of GTX-III following H_2O_2 application also caused a significant decrease in the viability of

Cos-7 cells at 24 h ($^*p < 0.05$). However, despite a decrease in cell viability at 48 and 72 h, no significant difference was found between the GTX-III groups and the control. On the other hand, no significant difference was found in the DMEM+GTX-III groups when compared to the control. This result showed us that GTX-III had no effect on healthy Cos-7 cells unlike H9c2 cells (Figure 2).

Figure 1. Impact of H_2O_2 and post treatment of GTX on the % viability of H9c2 cells and GTX treatments on the H9c2 cells without any treatment, after incubation periods (24, 48, and 72 h) (n = 6). (D1: 0.5 µM; D2: 1 µM; D3: 10 µM) ($^*p < 0.05$, $^\#p < 0.01$, $^+p < 0.05$)

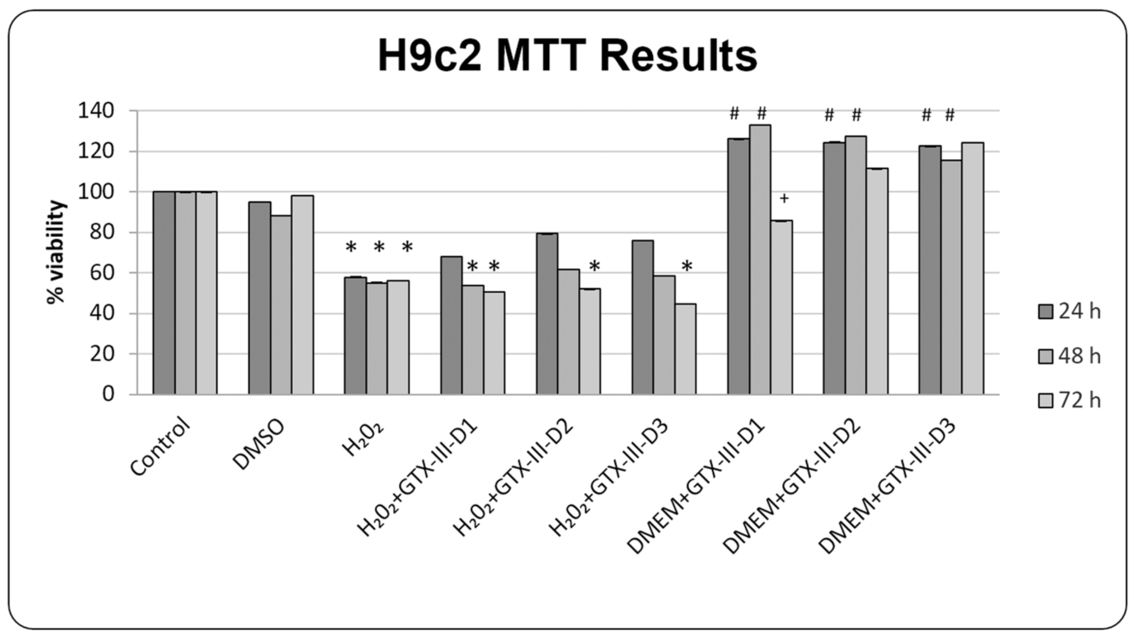
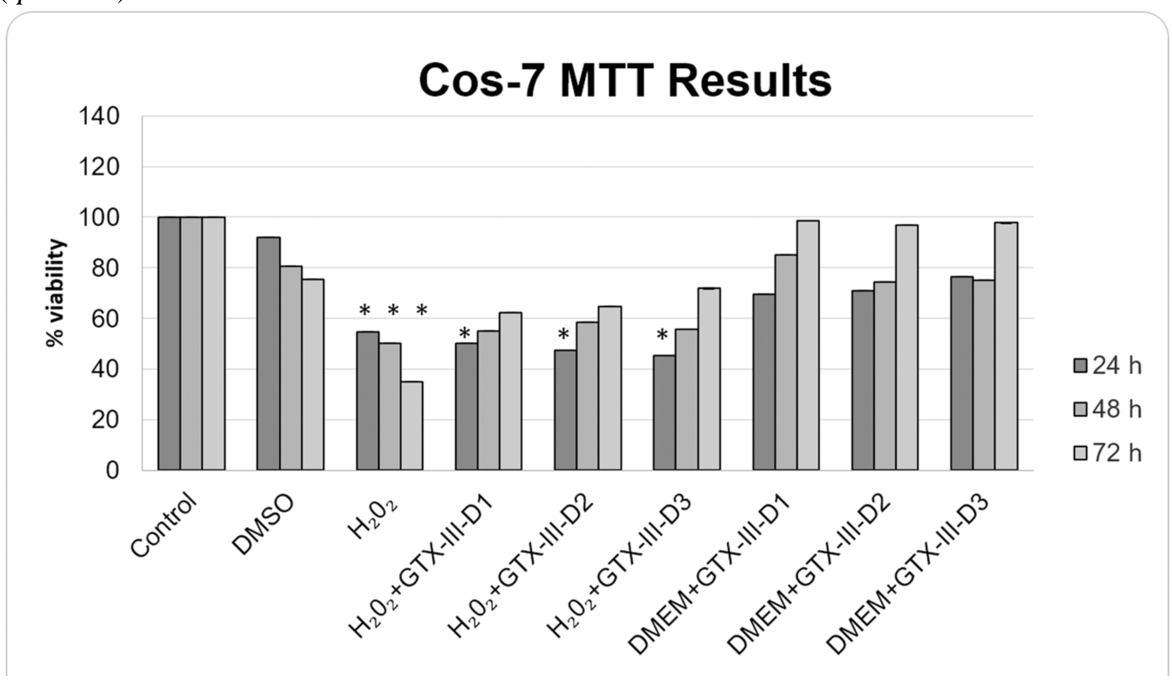


Figure 2. MTT results of Cos-7 cells after treatment with H_2O_2 and GTX's different concentrations for 24, 48, and 72 h. Results are derived from at least three replicates. (D1: 0.5 µM; D2: 1 µM; D3: 10 µM) ($^*p < 0.05$)



3.2. Apoptotic Staining Results

Results in H9c2 and Cos-7 cells showed an increase in apoptotic cell death in both cases after all incubation times at all concentrations of GTX-III used after H_2O_2. As shown in Figure 3-4, more apoptotic cells were observed in the H_2O_2 and H_2O_2-GTX-III groups than in the control and DMEM-GTX-III groups. According to these results, GTX-III has a protective effect against non-H_2O_2-induced cell apoptosis, while inducing apoptosis in H_2O_2-induced cardiomyocytes and renal cells.

When the percentages of apoptosis and necrosis in H9c2 cells were analysed, it was found that the H_2O_2 and H_2O_2+GTX-III groups showed a significantly higher percentage of both. The significant difference found was valid in all time periods. In the DMEM groups, there was no difference in all time periods in terms of both % apoptosis and % necrosis.

As a result of the analysis of Cos-7 cells, there was a significant increase in % apoptosis results of H_2O_2 and D2 and D3 concentrations of H_2O_2+GTX-III at 24th h compared to the control ($^*p < 0.05$). In addition, it was determined that there was a significant increase in the percentage of necrotic cells in the D2 concentration of DMEM+GTX-III ($^+p < 0.01$). A significant increase in both % apoptosis and % necrosis results was observed at 48 h for H_2O_2 and all concentrations of H_2O_2+GTX-III when compared to the control ($^*p < 0.05$, $^+ p < 0.01$). At the 72nd h, a significant increase was observed in % necrosis results in D3 concentration of H_2O_2+GTX-III and H_2O_2 when compared to the control ($^+p < 0.01$), while a significant difference in % apoptosis was observed only in D1 concentration of DMEM+GTX-III groups ($^*p < 0.05$) (Figure 5).

3.3. Lactate Dehydrogenase Assay Results

The LDH test revealed that cytotoxicity was significantly increased in H9c2 cells in H_2O_2 and in all GTX-III groups compared to the control group at 48 and 72 h ($P < 0.05$). LDH cytotoxicity was increased in a dose-dependent manner, representing an increase in the number of dead cells (Table 1). However, the small increase in all DMEM-GTX-III groups was not significant when compared with the control. Also, it was observed that increasing GTX-III doses showed a decrease in cytotoxicity effect over time. The maximum LDH value was observed in the 0.1 μM dose group of GTX-III at 48 and 72 h, whereas the viability percentage was observed to have the lowest percentage at 48 and 72 h (Figure 6).

Figure 3. AO/PI staining for apoptosis potential detection after H_2O_2 induce and non-induce H9c2 cardiomyocytes treated with different concentrations of GTX-III after 24, 48, and 72 h. The images of cells are x20 magnifications.

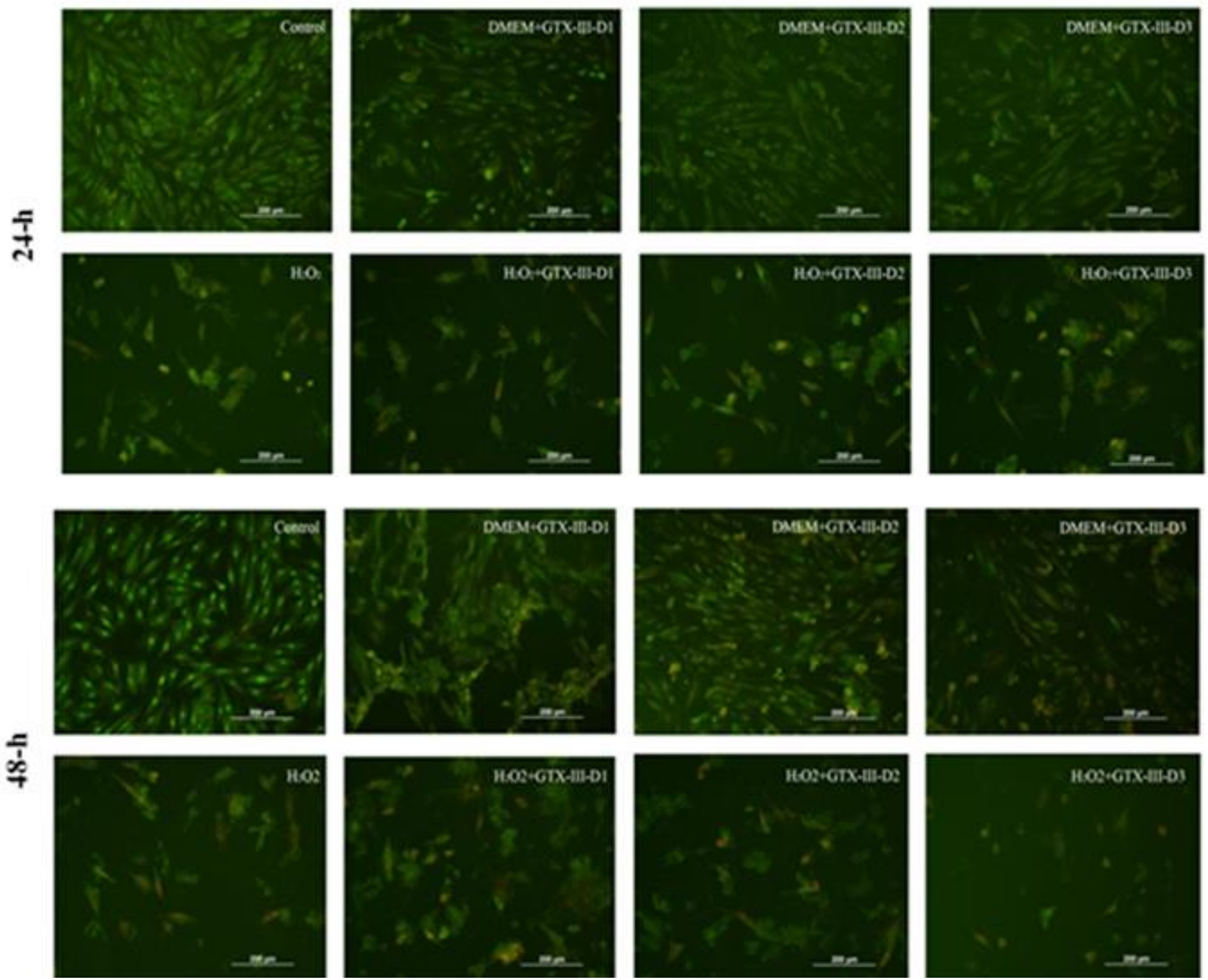


Figure 3. *Continues.*

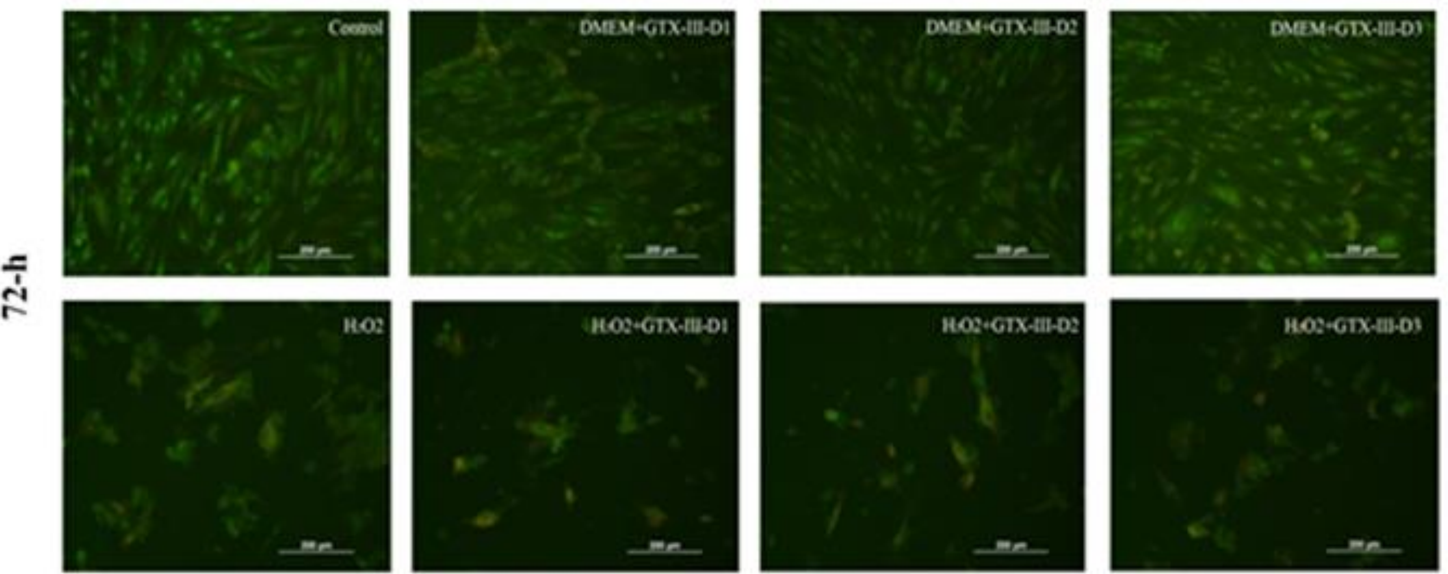


Figure 4. AO/PI staining for apoptosis potential detection after H_2O_2 induce and non-induce Cos-7 cells treated with different concentrations of GTX-III after 24, 48, and 72 h. The images of cells are x20 magnifications.

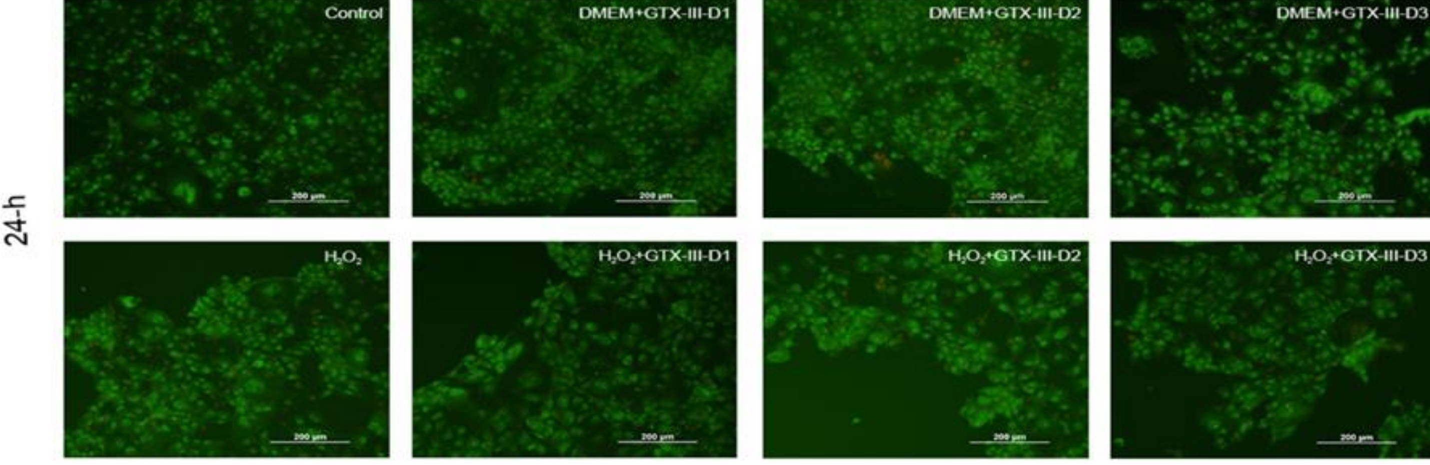


Figure 4. Continues.

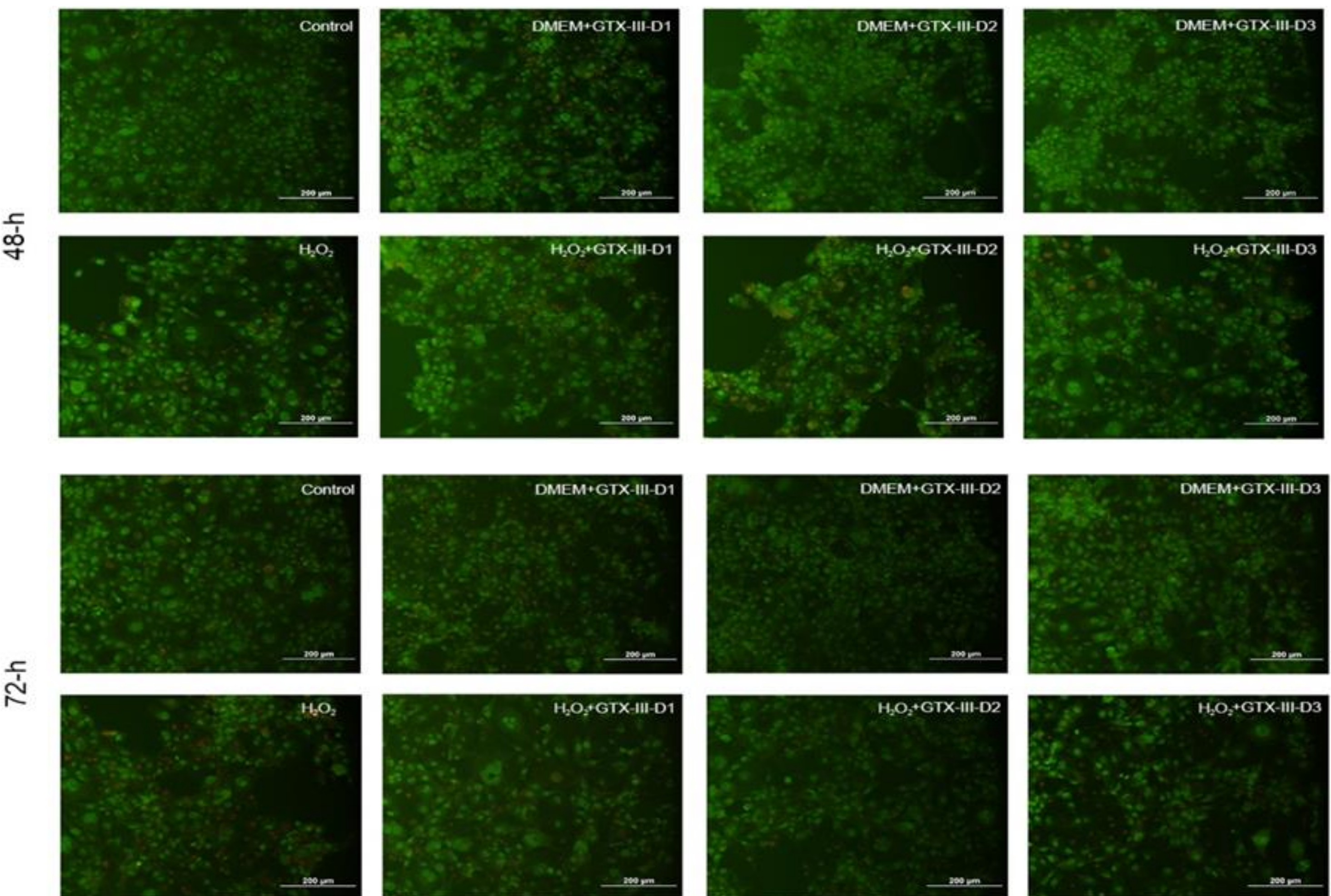


Figure 5. The % apoptotic and necrotic cells in H9c2 (A) and Cos-7 (B) treated with H_2O_2 and GTX's different concentration ($^{*}p < 0.05$, $+ \, p < 0.01$).

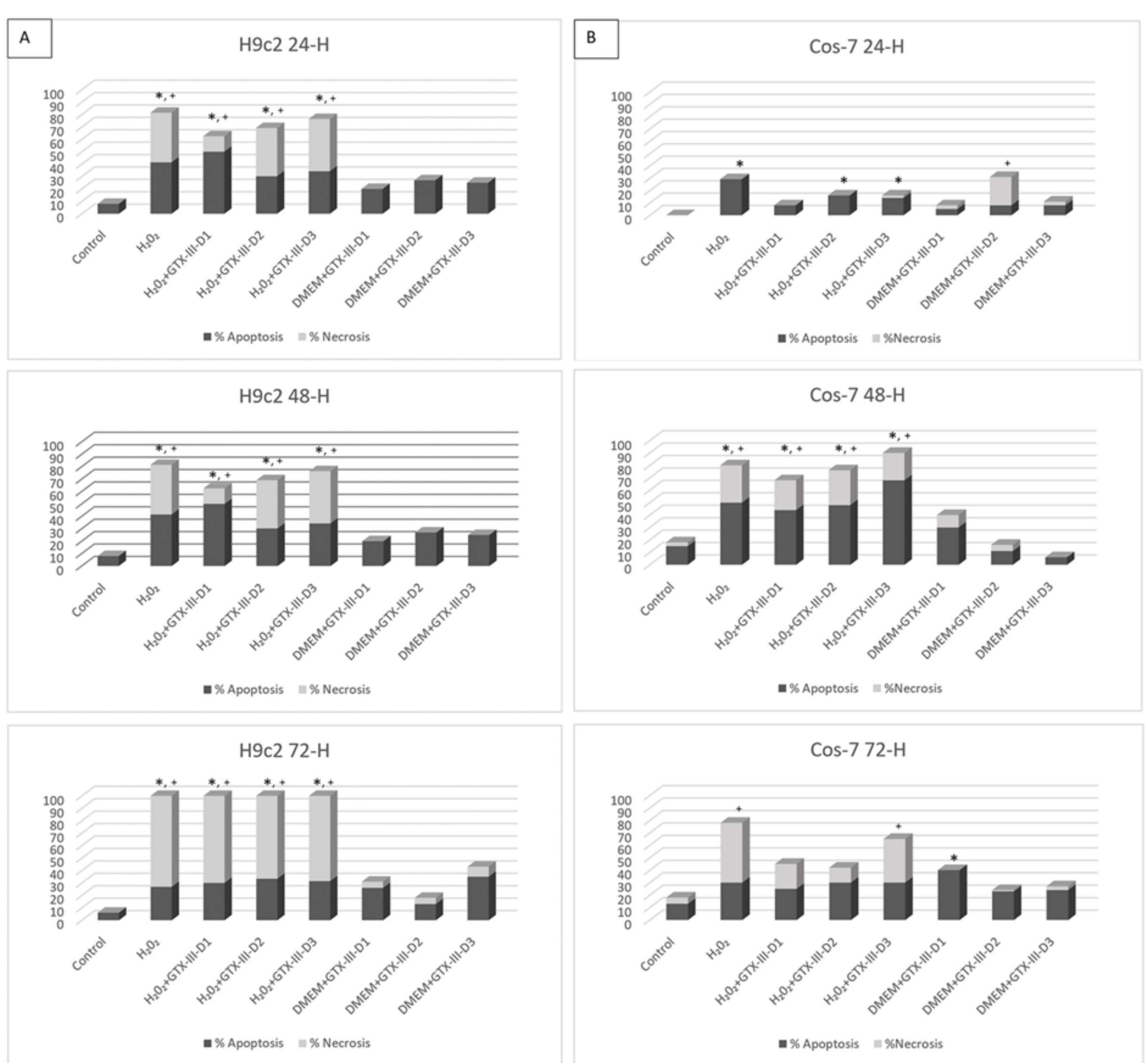


Table 1. Lactate dehydrogenase cytotoxicity assay results

	Control	H_2O_2	H_2O_2+ GTX-III-D1	H_2O_2+ GTX-III-D2	H_2O_2+ GTX-III -D3	DMEM+ GTX-III-D1	DMEM+ GTX-III-D2	DMEM+ GTX-III-D3
Cytotoxicity	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
48-hours	1.35±0.05	38.53±0.10	21.06±0.09	13.64±0.02	10.8±0.01	7.31±0.05	7.79±0.05	3.56±0.04
72-hours	2.38±0.02	37.22±0.12	13.40±0.00	8.37±0.00	8.63±0.02	5.76±0.02	5.00±0.09	3.37±0.02
p^*	0.026		0.027	0.028	0.035			

The results (Mean±SD) of three independent experiments are shown as percentages of the control.
D1: 0.5 µM; D2: 1 µM; D3: 10 µM; SD: Standard Deviation, *: Bonferroni post-tests

4. DISCUSSION and CONCLUSION

The current research aims to evaluate the potential dose-dependent effects of GTX-III on cardiomyocyte and renal cells in connection to the background of heart attack and renal failure. Within this frame, H9c2 and Cos-7 cells failed with an oxidative stress agent-H_2O_2.

Oxidative stress occurs when the balance between oxidants and antioxidants changes in favour of oxidants. The balance of reduction and oxidation events is critical for cell viability, activation, proliferation, and organ function. Oxidative stress formation causes the development of many pathological diseases (including cancer, neurological disorders, atherosclerosis, hypertension, ischemia/perfusion, diabetes, and asthma). Especially after coronary stenting, myocardial ischemia reperfusion injury (MI/RI) develops by triggering the pathways involved in oxidative stress damage and cell apoptosis. Myocardial or renal hypoxia and re-oxygenation cause an increase in many free radicals (superoxide radical (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (-OH) and peroxynitrite (ONOO-)) in tissues (Abdel *et al*., 2009; Elahi *et al*., 2009). Apoptosis or death ultimately results from lipid peroxidation of the cell membrane, the main mechanism of RI (Incalza *et al*., 2018). The change in oxygen free radicals triggered by MI/RI causes the initiation of a series of steps that induce cell death pathways associated with tissue damage. These damages are the observation of glucose and lipid peroxidation, enzyme inactivation, and protein denaturation, followed by DNA fragmentation. In short, oxidative stress-induced apoptosis is an important triggering factor in MI/RI development (Cakmak-Arslan *et al*., 2020). Our results indicate that GTX-III has a cytotoxic effect on the ischemia cell models, possibly by reduced activity in mitochondria. It was observed that GTX-III concentrations decreased cell viability in H9c2 and Cos-7 cells below 60% compared to control cells. Degeneration mostly involved acute cell swelling. Acute cell swelling is known to be more related to reduce cellular oxygenation (Elahi *et al*., 2009). When the oxygen used by the cell decreases with MI/RI, mitochondrial oxidative phosphorylation stops and the cells undergo anaerobic metabolism or die. If the cell fails to restore mitochondrial function, its entry into the path of death becomes irreversible and leads to cell necrosis (Wang *et al*., 2018). One of the markers of apoptotic cell death, necrotic cell death, was also observed in high-dose groups of GTX-III. It was observed that the DMEM-GTX-III-D1 group had the lowest % necrotic rate in all incubation periods in both cells. According to these results, GTX-III induces apoptosis in ischemic cardiomyocytes. It appears to have a protective effect against apoptosis in healthy cells. However, we cannot say the same for kidney fibroblast cells. According to the cell viability results, it was observed that the viability rate approached the control in healthy fibroblasts only at 72 h. It was also observed that increasing doses of GTX-III showed an increase in cytotoxicity over time in ischemic cardiomyocytes but decreased over time in fibroblast cells.

Based on the fact that LDH results support the cell viability, results strengthen the confirmatory aspect of our hypothesis. LDH test was used in this study to determine the cell

membrane damage of the GTX-III dose effect on damaged cardiomyocytes. And results showed that the increase in the number of dead cells was dose-dependent.

All the tested doses of GTX-III induce cell proliferation in healthy H9c2 cells. This can be attributed to various reasons. Kühn *et al*. (2007) reported that PI3K/Akt pathway induces the re-entry of cardiomyocytes into the cell cycle. In another study, gap junctional coupling was reported to be an important factor in cardiomyocyte proliferation (Weeke-Klimp *et al*., 2010). The factors that trigger these have not been studied in detail. A commonly known feature of GTX-III is that it eliminates rapid sodium inactivation and channel activation causes a hyperpolarized shift in voltage dependence. It has also been reported to be a lipid-soluble toxin that exhibits a selective property on voltage-dependent sodium channels (Maejima *et al*., 2003). With these in mind, GTX-III may have a potential inductive effect on different membrane proteinsor may have a secondary effect on activating certain pathways in cardiomyocytes.

DMEM-GTX-III was observed to cause aggregation in healthy cardiomyocyte cells at 48 and 72 h, especially at D1 concentration. This cellular behaviour, specifically observed in these cells in particular, shows that the use of GTX-III at this dose promotes the aggregation movement of cardiomyocytes and may trigger pulse observation after some time.

Due to their use in folk medicine, these species have become the focus of interest for scientific studies. Medicinal use of this product without obtaining any information about the consumption dose of a natural product makes it a real risk factor. While the in vivo effects of GTX-III have been examined in almost all of the previous studies, the important feature of this study is that GTX-III is examined at the cell level *in vitro* model of the ischemia-reperfusion injury cell culture model (Maejima *et al*., 2003; Xu *et al*., 2016). Also, previous cell culture studies with plant extracts and GTXs were generally performed with different cancer cell lines (Doğanyiğit *et al*., 2019). However, there have not (yet) been any studies on heart muscle and kidney fibroblast cells. To the best of our knowledge, this is the first study to document the cytotoxic properties and apoptotic potential of GTX-III, the main substance of commonly consumed mad honey in the *in vitro* cell culture model.

This study's primary limitation was that it only included *in vitro* experiments and no animal or human research. Also, it was done with a single isoform of the GTX-III. It should give more valuable information if the other isoforms were also evaluated. But on the other hand, using a pure GTX-III is the main strength of our study.

In conclusion, it was revealed that GTX-III induces cell proliferation in healthy H9c2 cells whereas promotes apoptosis in H_2O_2–induced cell death H9c2 cardiomyocytes. In addition, it was observed that GTX-III supports proliferation at a lower rate in healthy Cos-7 cells than in cardiomyocyte cells. Our results may provide a theoretical basis for dose-dependent alternative use of GTX-III in humans without heart and kidney disease.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Esin Akbay Çetin: designed the study and supervised the experiments **Çiğdem Özenirler**: made contributed in the analysis of the results. All these authors have substantial contributions to the final manuscript and approved this submission.

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Antimicrobial evaluation of the Patchouli (*Pogostemon cablin* Benth.) leaf essential oil combination with standard antimicrobial compounds

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Abstract: *Pogostemon cablin* Benth's leaves, which belong to the Lamiaceae family, are used to make patchouli essential oil (PEO). Sesquiterpenes are the main compounds in essential oil. Antibacterial, antifungal, antiviral, antiemetic, and anti-inflammatory activities are just a few of the many pharmacological actions that are well-known. In this investigation, cefuroxime (CEF), moxifloxacin (MOX), clarithromycin (CLA), fluconazole (FLU), and terbinafine (TER) were combined with patchouli (*Pogostemon cablin* leaf essential oil). Gram-positive *Staphylococcus aureus* ATCC 6538, Gram-negative *Escherichia coli* ATCC 8739, and yeasts *Candida albicans* ATCC 10231, and *C. tropicalis* ATCC 750 were tested against all combinations. Drug interaction was given as fractional inhibitory concentrations (FIC) after combinations of the essential oils were tested for their minimum inhibitory concentrations (MIC).

The checkerboard method was used to assess antimicrobial interactions, Fluconazole, terbinafine, cefuroxime, and clarithromycin combined with essential oil demonstrated an "additive effect" against *Candida* strains together with *E. coli* and *S. aureus*.

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1. INTRODUCTION

In modern medicine, antimicrobial substances especially antibiotics have been used for the protection of global health for the last eighty years (Laxminarayan *et al.*, 2016). With the discovery and development of antibiotics over time, it has been seen that infections can be controlled and prevented. However, the overuse and misuse of antibiotics used against microorganisms have led to the formation of antimicrobial resistance in microorganisms (Kapoor *et al.*, 2017). Antibiotic resistance, in addition to being a multi-sectoral problem that destroys decades of progress in medicine, food safety, and public health, causes very high rates of death worldwide, regardless of the level of development of countries (Laxminarayan *et al.*, 2016). Due to the increase in antimicrobial-resistant microorganisms and the inability to produce new antimicrobials easily, alternative strategies must be found to combat infections caused by drug-resistant organisms (Ahmad *et al.*, 2021; Langeveld *et al.*, 2014).

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Synergistic, additive, indifferent, and antagonistic effects can be produced when combining essential oils and antimicrobials. When the combined antimicrobial activity of the two compounds is larger than the combined antimicrobial activity of the two separately administered compounds, this is known as a synergistic effect (Göger *et al*., 2018). The synergic effect is thought to be caused by four different theoretical mechanisms, including blockage of a specific metabolic pathway, inhibition of enzymes that break down antimicrobials, interactions of some antimicrobials with the cell wall, and/or membrane interactions that result in an increase in the number of antimicrobial compounds taken up by the cell (Hyldgaard *et al*., 2012).

Essential oils exhibit a wide range of activities against pathogenic microorganisms. According to research that has been published, various combinations of essential oils and antibacterial compounds are efficient against microorganisms (Hemaiswarya & Doble, 2009; Van Vuuren *et al*., 2009; Göger *et al*., 2018; Sharma *et al*., 2020; Zhong *et al*., 2021).

Pogostemon cablin L., a plant belonging to the Lamiaceae family, is used mostly in traditional medicine in Southeast Asia (Dechayont *et al*., 2017). The word "cablin" is derived from the term "cabalam," which is also the local name for the patchouli plant in the Philippines and these are synonymous (Swamy & Sinniah, 2015). *P. cablin* has often been used in traditional medicine to promote digestion, restore function, and lower fever and chronic weariness (Dechayont *et al*., 2017; Picheansoonthon & Jerawong, 2001).

At least 140 biologically active chemicals, including terpenoids and flavonoids, are present in the significant medicinal plant *P. cablin* (Tang *et al*., 2019). There are several pharmacological qualities, including antibacterial, anti-inflammatory, antiplatelet, antioxidant, antithrombotic, aphrodisiac, analgesic, antimutagenic, antiemetic, antidepressant, fibrinolytic, and cytotoxic activity (Ribeiro *et al*., 2018; Swamy & Sinniah, 2015).

Patchouli (*Pogostemon cablin* L.) leaf essential oil (PCLO), which is derived from the leaves of *P. cablin*, has tremendous significance due to the structure of its twenty-four distinct sesquiterpenes (Donelian *et al*., 2009; Silva-Filho *et al*., 2016). Scientists have looked at patchouli essential oil's therapeutic qualities, including its cytotoxic, antioxidant, anti-inflammatory, antibacterial, and antidepressant effects (Aisyah *et al*., 2021; Jain *et al*., 2021; Paulus *et al*., 2020).

For this study, we combined *P. cablin* leaf essential oil with antimicrobial compounds such as, cefuroxime, moxifloxacin, clarithromycin, fluconazole, and terbinafine against strains of Gram-positive *Staphylococcus aureus* ATCC 6538, Gram-negative *Escherichia coli* ATCC 8739, and yeasts *Candida albicans* ATCC 10231, and *C. tropicalis* ATCC 750. To the author's knowledge, this is the first study to investigate the effects of combining essential oil of *Pogostemon cablin* with antimicrobial compounds.

2. MATERIAL and METHODS

2.1. Essential Oil

An amber glass bottle of PCLO was purchased from Bade Natural Ltd. in Istanbul, Turkey. The essential oil was kept in a 4°C refrigerator. Analyses of the essential oil supplied by the provider using Gas Chromatography (GC).

2.2. Materials

As conventional antimicrobial drugs, Sanovel Pharmaceutical Company (Istanbul, Turkey) provided the following: cefuroxime (CEF), moxifloxacin (MOX), clarithromycin (CLA), fluconazole (FLU), and terbinafine (TER). To determine antimicrobial activity and checkerboard microdilution assays, RPMI-1640 medium with L-glutamine and Mueller Hinton

Broth (MHB, Sigma) were used. RPMI medium was buffered to a pH of 7 with 3-[N-morpholino]-propane sulfonic acid (MOPS).

2.3. Antimicrobial Activity Studies

2.3.1. *Bacterial and Yeast strains*

The standard strains were *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Candida* albicans ATCC 10231, and *C. tropicalis* ATCC 750 from the American Type Culture Collection (ATCC).

2.3.2. *Determination of minimum inhibitory concentration* (MIC)

Susceptibility studies for PCLO were performed using microdilution methods adapted from CLSI guidelines (CLSI, 2002; CLSI, 2006). The essential oil was dilutedtwo-fold initially, with a final concentration range of (40-2560 µg/mL), for standard antibacterial drugs (0.125- 64 µg/mL), and antifungal drugs (0.25-128 µg/mL).

For the preparation of cell suspensions in MHB for bacterial strains and RPMI medium for yeasts, fresh overnight cultures of the tested microorganisms were employed to achieve 10^6 colony forming units (CFU)/mL and 1-2x10^3 cells/mL, respectively. The experiments were conducted in a 96-well plates, and inoculated microplates were incubated at 37°C for 24 hours for bacteria and 48 hours for yeast, respectively. Adding 15 µL of 0.01% resazurin allowed for the observation of microbial growth. MICs were established as the lowest concentrations of resazurin that did not result in bacterial or fungal growth or a color change.

2.3.4. *Combination of EO and standard antimicrobial compounds*

The antimicrobial interactions of the antibiotics (CEF, MOX, and CLA) and antifungals (TER, FLU) with PCLO were studied using the checkerboard method. The 10-to-7-well design was used on 96-well plate. The same solvents (media) used in the MIC test were used to generate seven serial dilutions of PCLO and ten dilutions of antimicrobial drugs (CEF, MOX, CLA, TER, and FLU).

In the first row A of a 96-well plate, 200 µL of PCLO were placed. Then the essential oil was serially diluted to the other six rows in horizontal direction. Similarly, 200 µL aliquots of each antimicrobial drug (CEF, MOX, CLA, TER, FLU) were added in a vertical (column) orientation, and serially diluted. Similarly, 200 µL of antimicrobial drugs CEF, MOX, CLA, TER, and FLU were introduced and serially diluted in vertical column orientation. Thus, various concentrations of essential oil and antimicrobial drugs were formed in the plate. The positive growth controls were used free of antimicrobial drugs in the wells. To evaluate sterility, negative growth controls were also established. Microorganisms were individually pipetted into all wells except for negative controls, with a McFarland value of 0.5 for each microorganism, 50 µL for bacteria, and 100 µL for Candida, respectively. Bacteria were incubated for 24 hours and candida for 48 hours at 37°C. Following incubation, 15 µL of 0.01% resazurin was added to each well and incubated for 2 hours at 37°C. The color of the medium changes from blue to pink as it grows.

The following formula (Van Vuuren *et al.*, 2009) was used to calculate the interaction of combinations based on the sum of the fractional inhibitory concentration (FIC):

$$\text{FIC of essential oil} = \frac{\text{MIC of essential oil in combination with antimicrobial drugs}}{\text{MIC of essential oil alone}}$$

$$\text{FIC of antimicrobial drug} = \frac{\text{MIC of antimicrobial in combination with essential oil}}{\text{MIC of antimicrobial drug alone}}$$

$$\text{FICI} = \text{FIC of essential oil} + \text{FIC of antimicrobial drug}$$

The impacts were classed as follows:

Table 1. The effects and ranges of combinations.

	Ranges	Effect
FICIs	≤0.5	synergistic
	0.5≤1	additive
	1–4	indifferent
	≥4	antagonistic

3. RESULTS

3.1. Specifications of *Pogostemon cablin* L. Leaf Essential Oil

The supplier at Bade Natural provided the analysis certificate for PCLO. Specifications for *Patchouli* essential oil was given in Table 2. The major components of PCLO were identified as patchouli alcohol (30.0–60.0%), guaiene (10.0–30.0%), and caryophyllene (1.0–5.0%) (GC) by using Gas Chromatography.

Table 2. Specifications for PCLO from Bade naturel.

Compounds	%
Patchouli alcohol	30.0 - 60.0%
α-guaiene	(10.0 - 30.0%)
β-caryophyllene	(1.0 - 5.0 %)
The density (at 20°C)	0.943-0.983 g/mL
Refractive index (at 20°C)	1.502-1.514

3.2. Minimum Inhibitory Concentrations (MIC)

The minimum inhibitory concentration values PCLO and standards were found for each microorganism and represented in Table 3. In this study, the MIC of PCLO in the range of >2560-640 µg/mL against bacteria and yeasts. The PCLO was found to be more effective against *Staphylococcus aureus* ATCC 6538 at MIC= 640 µg/mL than *Escherichia coli* ATCC 8739 at MIC= 2560 µg/mL. The MIC values were found at 1280-2560 µg/mL for *C. tropicalis* ATCC 750 and *C. albicans* ATCC 10231 strains, respectively.

Table 3. Minimum inhibitory concentrations (µg/mL).

Compounds	*E.coli* ATCC 8739	*S.aureus* ATCC 6538	*C.albicans* ATCC 10231	*C.tropicalis* ATCC 750
PCLO	>2560	640	2560	1280
CEF	64	64	-	-
MOX	0.25	0.25	-	-
CLA	64	0.25	-	-
TER	-	-	32	16
FLU	-	-	2	4

PCLO: *Pogostemon cablin* L. leaf essential oil- CEF: Cefuroxime, MOX: Moxifloxacin, CLA: Clarithromycin, TER: Terbinafine, FLU: Fluconazole

3.3. Combination of Essential Oil and Standard Antimicrobial Compounds

The checkerboard method was used to determine the antimicrobial interaction of antibiotics (CEF, MOX, and CLA) with PCLO. In the present study, the antimicrobial activity of *Patchouli* essential oil was tested on *S. aureus* and *E. coli* strains. While an additive effect was obtained with cefuroxime and clarithromycin, an indifferent effect was observed with moxifloxacin. According to the results of the combination, essential oil with cefuroxime and clarithromycin showed an additive effect (FICI=1.0) against *S.aureus* ATCC 6538. The essential oil in combination with moxifloxacin was defined as having an indifferent effect (FICI=1.25 for the same strain) (in Table 4).

The combination of essential oil with cefuroxime and clarithromycin showed additive effects (FICI=1.0), and (FICI=0.75), respectively, against *E. coli*. The combination of essential oil with moxifloxacin had an indifferent effect (FICI=1.25) against the same strain (in Table 5). The combination of the essential oil with fluconazole had an additive effect for both *Candida* strains, while the combination with terbinafine had an indifferent effect for *C.albicans* (in Table 6) and an additive effect for *C.tropicalis* (in Table 7). Briefly, our test organisms demonstrated neither synergistic nor antagonisticeffects.

Table 4. Combination of essential oil with antibiotics against *S. Aureus.*

Combinations	PCLO			Standard Comp.			FICI	Result
	Alone	Comb.	[1]FIC	Alone	Comb.	[2]FIC		
PCLO+ CEF	640	320	0.5	4	2	0.5	1	Additive
PCLO+ MOX	640	160	0.25	0.031	0.031	1	1.25	Indifferent
PCLO+ CLA	640	320	0.5	0.5	0.25	0.5	1	Additive

PCLO: *Pogostemon cablin* L. leaf essential oil - CEF: Cefuroxime, MOX: Moxifloxacin, CLA: Clarithromycin

Table 5. Combination of essential oil with antibiotics against *E. coli.*

Combinations	PCLO			Standard Comp.			FICI	Result
	Alone	Comb.	[1]FIC	Alone	Comb.	[2]FIC		
PCLO + CEF	20480	10240	0.5	32	16	0.5	1	Additive
PCLO + MOX	10240	2560	0.25	0.031	0.031	1	1.25	Indifferent
PCLO + CLA	10240	5120	0.5	256	64	0.25	0.75	Additive

PCLO: *Pogostemon cablin* L. leaf essential oil- CEF: Cefuroxime, MOX: Moxifloxacin, CLA: Clarithromycin

Table 6. Combination of essential oil with antifungals against *C. albicans.*

Combinations	PCLO			Standard Comp.			FICI	Result
	Alone	Comb.	[1]FIC	Alone	Comb.	[2]FIC		
PCLO + TER	2560	640	0.25	8	8	1	1.25	Indifferent
PCLO + FLU	2560	40	0.0157	1	0.5	0.5	0.5157	Additive

PCLO: *Pogostemon cablin* L. leaf essential oil- TER: Terbinafine, FLU: Fluconazole

Table 7. Combination of essential oil with antifungals against *C.tropicalis.*

Combinations	PCLO			Standard Comp.			FICI	Result
	Alone	Comb.	[1]FIC	Alone	Comb.	[2]FIC		
PCLO + TER	2560	1280	0.5	32	4	0.125	0.625	Additive
PCLO + FLU	2560	1280	0.5	1	0.031	0.031	0.531	Additive

PCLO: *Pogostemon cablin* L. leaf essential oil- TER: Terbinafine, FLU: Fluconazole

4. DISCUSSION and CONCLUSION

According to published research, the chemical composition of patchouli oil from Samia Aromatherapy was revealed to contain patchoulol (25.21%), *δ*-guaieno (11.49%), *α*-gurjunene (11.26%), seicheleno (9.61%), *α*-guaieno (9.56%), benzyl alcohol (6.73%), vidreno (3.12%), aromadendrene (2.81%), *α*-cedrol (2.63%), and *β*-patchouleno (1.57%). The density value was found to be 1009 mg/mL (Murbach Teles Andrade *et al.*, 2014).

The Chinese Pharmacopoeia defines the essential oil of patchouli, which is derived from the leaves of *Pogostemon cablin* (Lamiaceae) (Pharmacopoeia, 2015). The characteristics of patchouli essential oil include its color, scent, specific gravity (between 0.95 and 0.98), specific rotation (43°-66°), and refractive index (1.503 and 1.513). Patchoulol should have a minimum content of 26.0%. (Pharmacopoeia, 2015; van Beek & Joulain, 2018).

In the previously published study, the patchouli essential oil was compared by three different detectors on five different GC systems for the analysis of patchoulol using internal standardization and found absolute percentages at 31.0, 30.6, 30.9, 30.7, and 30.9% (van Beek & Joulain, 2018). Patchouli essential oil often displayed a variety in its chemical composition, but these findings are consistent with the literature.

Yang and colleagues reported that the antimicrobial activity of *Patchouli* essential oil MIC values found between 1.0 to 6.5 mg/mL for *E. coli* and *S. typhi*, respectively (Yang *et al.*, 2013). However, Das and colleagues reported MIC between the range 250 to 1000 µg/mL in their study against nine bacterial strains (Das *et al.*, 2013). Andrade *et al.* reported *the* antimicrobial activity of *P. patchouli* essential oil against *S. aureus*, *E. coli*, and *P. aeruginosa.* The minimum inhibitory concentration was defined as MIC_{50} and MIC_{90} values. The results of essential oil were found $MIC_{50}=0.25$, $MIC_{90}=0.25$ for *Staphylococcus aureus*, ($MIC_{50}\geq30.27$ and $MIC_{90}\geq30.27$) for *E. coli*, and ($MIC_{50}>30.27$, $MIC_{90}\geq30.27$) *P. aeruginosa*, respectively (Murbach Teles Andrade *et al.*, 2014).

In another study, Singh et al (2019) reported that the antimicrobial activity of *Patchouli* essential oil was tested against clinically important 80 bacterial isolates. The results of the study Gram-positive bacteria were significantly more sensitive to *Patchouli* essential oil than Gram - negative bacteria. These findings are in agreement with our study.

Kocevski et al (2013), the antifungal activity of *Allium tuberosum*, *Cinnamomum cassia*, and *Pogostemon cablin* essential oils were investigated on *Aspergillus flavus* and *Aspergillus oryzae* According to these data, it was reported that *A. tuberosum* and *C. cassia* essential oils inhibited *A. flavus* 32758 and 34408, and *A. oryzae* with a MIC 250 ppm, while patchouli essential oil was found to be > 1500 ppm. The essential oils showed an inhibitory effect on colony growth at 100, 175, and 250 ppm for *A. tuberosum*, and was8 found to be 25, 50, and 75 ppm for *C. cassia*. Also, the essential oil *P. cablin* showed inhibitory an effect at 100, 250, and 500 ppm (Kocevski *et al.*, 2013).

When compared to the combined effects of the individual compounds, the essential oils and other components possess particular benefits for antimicrobial activity (Seow *et al.*, 2014). Their mechanism of action has multiple targets on microorganisms, including cell wall destruction, increasing permeability, leakage of the cell content, damaging of the cytoplasmic membrane, membrane protein damage, and coagulation of cytoplasm resulting in metabolic damage and cell death (Betoni *et al.*, 2006; Bhavaniramya *et al.*, 2019).

To the best of our knowledge, this is the first study for a combination of *Pogostemon cablin* L. leaf essential oil with antimicrobial drugs. Various combinations demonstrated mainly additive interactions between Patchouli essential oil and antimicrobial agents. The antagonistic effect did not found with *E. coli, S. aureus,* or *Candida* strains. This is probably due to their being generally more sensitive to the effects of essential oil components. The antagonistic effect

was not observed with the strains of *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *C. tropicalis* ATCC 750, and *C. albicans* ATCC 10231. The absence of antagonistic effects in this study suggests that patchouli essential oil has the potential to enhance the antimicrobial activities of other antibacterial drugs for a synergistic antimicrobial impact against various resistant pathogens.

In a study, the antimicrobial activity of patchouli, citronella, and nutmeg essential oils and these oil mixtures were determined against *S. aureus*, *Shigella sp.*, *C. Albicans,* and *Aspergillus niger* by disc diffusion method. The patchouli oil was found to be active against *S. aureus* (z.i=11.36 mm), and *Shigella sp* (z.i= 6.80 mm), respectively. In addition, when compared to citronella and nutmeg oils, patchouli oil was found to be only efficient against *Shigella* sp. Additionally, it was discovered that the patchouli and citronella oil mixtures created a greater inhibitory zone on *S.aureus* than gentamicin. On the other hand, patchouli oil was found to generate an inhibition zone when combined with other oils, however, when applied alone, it had no antifungal impact on *C. albicans* and *A. niger* (Aisyah *et al.*, 2021).

As a result, the current study's findings appear promising and may expand the usage of natural products, demonstrating the potential of this plant essential oil in the treatment of infectious disorders caused by *Staphylococcus aureus*. Future research should be done on the chemical properties of extracts and active ingredients for plants and their antibacterial activities.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

Authorship Contribution Statement

Nazlı Şenay Beşirik: Investigation, and Formal Analysis. **Gamze Göger:** Investigation, Methodology, Resources, Visualization, Software, Formal Analysis, Supervision, and Writing original draft.

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Physicochemical evaluation to assess the quality of honey samples marketed in Oman

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Abstract: The study aimes to investigate the physicochemical properties of seven honey samples to assess their quality as per GCC Standardization Organization (GSO) and international standard parameters. Seven honey samples, four marketed honey samples, and three locally produced Omani honey were collected and analysed for the pH, acidic content, % of insoluble matter, moisture content, proline, hydroxyl methyl furfural (HMF) and total reducing sugar contents. The results showed that pH of the tested honey samples are within the limit however acidity of the three samples did not comply with the prescribed limits. The moisture, proline, and hydroxy methyl furfural (HMF) contents of the honey samples tested are found to be within the acceptable range. However, the % of insoluble matter expressed for locally produced Sidr, Sumer, and Zah'r honey samples was below the maximum limit (0.5%) while marketed honey samples exceeded the limits of GSO (0.1%). The total reducing sugar concentration was below the limit in terms of four samples. Most of the tested honey samples meet the International/GSO standards for quality while a few failed to comply with acidity limits, the total reducing sugars content, and % of insoluble matter.

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1. INTRODUCTION

Honey, characterized by its sweet taste, is a natural product that does not contain artificial substances. It is a semi-liquid sugar solution that is used as a popular substitute for sugars. Honey is collected by bees from the nectar from flowers and plants and stored in combs. Honey has been known since ancient times for its nutritional and therapeutic properties (Ahmed *et al.*, 2014; Tariq *et al.*, 2022). Honey is a composition of sugars (mainly glucose and fructose) as well as 20% of water and a small percentage of proteins, enzymes, vitamins, minerals, amino acids, and volatile compounds (Mokaya *et al.*, 2020). Honey is rich in many health-promoting nutrients, which are carbohydrates, potassium, iron, and zinc (Moniruzzaman *et al.*, 2013). It has therapeutic properties *i.e.,* anti-fungal, anti-bacterial, and anti-inflammatory, and is also a powerful natural antioxidant. In Oman, Honey is produced by two types of bees, *Apis florea* (Omani Dwarf bees) and *Apis mellifera* (Omani domesticated bees). The good quality among the different varieties of honey is Al-Baram or Al-Sumer (*Acacia tortilis*) and Sidr (*Ziziphus*)

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honey (Shafeeq, 2016). The composition of honey varies according to the diversity of bees, the region, the season, the source of the nectar, as well as the method of harvesting and storage (Al-Farsi *et al*., 2018). As honey is an essential daily-life dietary supplement, it is important to evaluate its quality to ensure its purity and to make sure that it has all nutritional components present in it. This quality control evaluation can be done by assessing physicochemical parameters such as the pH, moisture, ash value, hydroxyl methyl furfural (HMF) and colour, etc., to evaluate and test the purity of marketed honey (Gela *et al*., 2021; Lewoyehu & Amare, 2019; Sabir & Mohammed, 2011). The pH and the total acidity measure the quality of the honey product and pH is not directly related to the acidity due to buffering action by the minerals and acids present in the honey (Singh & Singh, 2018). Moisture test helps in determining water content inside honey components which affects honey stability against fermentation and granulation. In addition, moisture testing can help the honey producer to maintain the proper storage and transport conditions (Sereia *et al*., 2017). Ash content which is another physicochemical parameter is used to evaluate richness of the honey in minerals content and it is widely used in nutritional aspects (Boussaid *et al*., 2018; Tigistu *et al*., 2021). Honey contains a lot of bioactive compounds including, phenols, flavonoids, vitamins, organic acids, and carotenoids, that may potentiate the antioxidant effects of honey (Mokaya *et al*., 2020). The bioactive compounds showed antioxidant activity by the mechanism of either scavenging free radicals, quenching singlet oxygen, or chelation of the radicals or metal ions. Honey is used since ancient times for both domestic consumption and fulfilling medical needs. The rich antioxidant properties of honey due to its high phenolic contents, flavonoids, and presence of catalase, glucose oxidase, ascorbic acid, organic acid, amino acids, and carotenoids derivatives make it more popular recently as a source of antioxidants. The honey a rich source of antioxidants works both orally or topically as a cough suppressant, to treat burns and infections. It can be used for immune boosting, antimicrobial, and relieving stress and anxiety. It has a preventive role in the progress of neurological diseases, cancers, cardiovascular diseases, and aging (Ratiu *et al*., 2020). The current research work involves the evaluation of the physicochemical parameters in the marketed honey and verifying their compliance with GSO standards. The evaluation of different quality control parameters was performed according to the Harmonised methods of the International Honey Commission (Afshari *et al*., 2022; Bogdanov *et al*., 2002; Rysha *et al*., 2022).

2. MATERIAL and METHODS

2.1. Collection of Honey Samples

A total of seven honey samples were collected from different groceries, supermarket, and hypermarkets in Oman. The list of samples coded as AFS1 to AFS7 and presented in Table 1, which shows the location, and type of honey. The honey was evaluated for moisture contents, pH, insoluble matter, proline contents, HMF, and total reducing sugars values. Each experiment was repeated thrice. All the chemicals including formic acid, ninhydrin and the proline used were of analytical grade, and purchased from Thermo Scientific chemicals.

Table 1. Code, location, and the marketed honey samples.

S. No	Code Assigned	Types	Location
1	AFS1	Pure Australian honey	Al-Batinah North -Liwa Hypermarket
2	AFS2	Natural bee honey Saudi Arabia	Al-Batinah North -Liwa Hypermarket
3	AFS3	Pure bee honey Dubai	Al-Batinah North -Liwa Hypermarket
4	AFS4	Sumer (*Acacia tortilis*)	Ad-Dakhiliyah Governorates
5	ASF5	Sidr (Ziziphus)	Ad-Dakhiliyah Governorates
6	ASF6	Zah’r (Flower)	Ad-Dakhiliyah Governorates
7	ASF7	Australian origin honey	Al-Batinah North -Liwa Hypermarket
	Total Samples		07

2.2. Measurement of Moisture Content of Samples

A refractometer is used to measure the refractive index (RI) and Brix at 20°C, from which the humidity value is calculated. Moisture content is defined as the amount of water present in honey which affects honey stability against fermentation and granulation. The high-water content in honey is related to its storage and harvesting, as the high percentage of moisture leads to the growth of mould and yeast, decreases the shelf life, and loss flavour. It is measured according to the International Honey Commission (IHC) (Bogdanov *et al*., 2002). The susceptibility to honey increases towards microorganisms with water contents above 17%(Puścion-Jakubik *et al*., 2020). Lower moisture contents (20%) increase the shelf life of the honey(Al-Farsi *et al*., 2018). The Abbe refractometer was used (Bellingham + Stanley Ltd. Abbe 60/DR refractometer) to calculate the moisture contents in the tested honey sample taking water as the reference material. The sample was homogenised and was heated in a water bath at 50 °C to dissolve honey crystals, if any. It was Cooled and stirred again at room temperature. The prism surface was covered with the sample and the refractive index (RI) reading was determined (Bogdanov *et al*., 2002). The temperature correction i.e., the refractive index was calculated at 20 °C using the following equation.

RI at 20 °C = RI at measured temperature (T) + 0.00045 (T-20 °C) provided T is above 20 °C.

2.3. Measurement of pH of Samples

The pH expresses the concentration of hydronium in honey and it can affect further the percentage of HMF contents. The most common organic acids present in the honey samples are citric and gluconic acid and others (malic, butyric, lactic, formic, acetic, and succinic acid, etc). The pH analysis is useful to estimate the quality of the honey. The increase in pH may indicate fermentation or adulteration (Al-Farsi *et al*., 2018).

Acidity by the titrimetric method: The variation in the content of some organic acids and phosphate (inorganic ions) depends on different sources of nectar, acidity, and the activity of the enzyme glucose oxidase. The increase in acidity affects the development of yeast and mould in the honey product. Free acidity should not be more than 50 milliequivalent/kg of honey (Puścion-Jakubik *et al*., 2020). A pH meter was calibrated to pH 4, 7, and 10. 10 g of the honey sample was dissolved in 75 mL of water. It was stirred with the help of a magnetic stirrer and then a pH electrode was immersed in it to measure the pH directly (Digital pH meter Martini instruments). The solution was slowly titrated with 0.1 M NaOH until the pH reached 8.30. The reading was recorded, and free acidity was calculated in meq/kg. The experiment was performed in triplicate.

2.4. Determination of Insoluble Matter of Samples

The insoluble matter may be present in the final product during preparation and indicate how well the honey is strained during extraction from the honeycombs or during processing and packaging. The honey sample should not contain more than 0.1 g/100 g (0.1% w/w) of insoluble ingredients except for pressed honey where it should be no more than (0.5% w/w)(Puścion-Jakubik *et al*., 2020). In this 20 gm of honey was dissolved in 200 mL of water at 80 °C. It was filtered with a dried crucible and washed thoroughly with warm water until it was free from sugars. The residue left was dried at 135 °C to a constant weight.

Calculation: The % insoluble matter calculated as g/100 g = $m/m_1 \times 100$

Where, m = mass of dried insoluble matter

m_1 = mass of honey taken

2.5. Proline Content

The proline content is used to check honey ripeness. It is the major amino acid present in bee honey. The amount of proline present in the honey sample is determined by the formation of the coloured complex with the ninhydrin and is directly related to the reading of absorbance at 510 nm as per the Ough method of Proline determination in honey (Bogdanov *et al*., 2002; Ough, 1969). The proline content should not be less than 25 mg/100g (Puścion-Jakubik *et al*., 2020). Honey with a proline content of less than 180 mg/kg is considered unripe in Germany (Bogdanov *et al*., 2002). A 0.5 mL of the honey sample was taken in a test tube (5g in 50 mL of water and then dilution it to 100 mL) and 0.5 mL of water (blank) in the second test tube and 0.5 mL of proline standard (stock solution 40mg/50mL and 1 mL diluted to 25mL to get 0.8mg/25mL) in three other test tubes. In each test tube, 1 mL of ninhydrin and 1 mL of formic acid were added. After shaking it was placed in a boiling water bath for 15 min and remained in the water bath for another 10 minutes at 70 ^{o}C. In all the test tubes, 5 mL of isopropanol and water were added. The absorbance of the sample and standard was measured at 510 nm using a UV spectrometer (Spectrum Instruments SP-UV 500DB spectrophotometer) (Bogdanov *et al*., 2002).

Calculation: The proline content (mg/kg) in honey sample is calculated from the formula:

$$\frac{Es}{Ea} \times \frac{E1}{E2} \times 80$$

Es = Absorbance of the sample solution

Ea = Absorbance of proline standard solution (average)

E_1 = mg proline taken for standard solution

E_2 = weight of honey in grams

80 dilution factor

2.6. Hydroxy Methyl Furfural (HMF) Content

The hydroxy methyl furfural (HMF) is formed due to an increase in the temperature and during the reaction of dehydration of sugar. It results from the Millard reaction (results from the reaction of reducing sugar and amino acids to form complex compounds). It is determined by taking the difference in the UV absorbance of the clear aqueous honey and the same honey sample after the addition of bisulphite (to avoid interference from other components) at 284 nm. The value is then subtracted from the background absorbance at 336 nm. Honey for sale should not contain more than 40 mg/kg (Puścion-Jakubik *et al*., 2020). The 5g of the honey sample was dissolved in 25 mL of the water and 0.5 mL of Carrez solution I (15 g of potassium hexacyanoferrate (II) in 100 mL of water) and 0.5 mL of Carrez solution II (30 g of zinc acetate in 100 mL of water) was added and made up the mark to 50 mL with water. The first filtered 10 mL was rejected and the next 5 mL each in two test tubes (I and II) were taken. Then 5 mL of water was added to test tube I (sample solution) and 5 mL of the sodium bisulphite solution (0.2% freshly prepared) to test tube II (reference solution). The absorbance of the sample solution against the reference solution was measured at 284 and 336 nm, respectively using a UV spectrophotometer (Spectrum Instruments SP-UV 500DB spectrophotometer) (Bogdanov *et al*., 2002).

Dilution, D = Final volume of the solution/10

Calculation: HMF (mg/kg) = $(A_{284} - A_{336}) \times 149.7 \times 5 \times D/W$

D = dilution

W = wt. in g of the honey sample

2.7. Total Reducing Sugars Determination by Titration Method

The sugars present in honey are mainly fructose, glucose, and low concentration of other sugars such as sucrose and maltose. The Fehling A and B titration method was used for estimating the total reducing sugars. Reducing sugar reduces Fehling's solution. The titration methods were used for the determination of glucose using methylene blue as an indicator (De Beer *et al*., 2021; Puścion-Jakubik *et al*., 2020). However, according to the GSO standard, the total reducing sugars present in the honey samples should be above 45g/100g (Al-Farsi *et al*., 2018). The carbohydrate concentration was used for the estimation of botanical origin and its proper classification(Puścion-Jakubik *et al*., 2020). Lane and Eynon's method was used to determine the total reducing sugar contents of honey (LANE, 1923). Fehling's solution A (7 g of $CuSO_4$. $5H_2O$ in 100 mL of distilled water) and Fehling's solution B (35 g of potassium sodium tartrate in 12 g of NaOH diluted to 100 mL distilled water) were freshly prepared. A standard inverted sugar solution was prepared. Briefly, the burette was filled with standard inverted sugar solution (0.985 g dried sucrose dissolved in 500 mL of water, then added 2 mL of concentrated sulphuric acid). The solution was boiled for 30 minutes and kept for 24 hours). After a day, it was neutralized with sodium carbonate, and the final volume was made up to 1000 mL. A blank titration was performed.

In a 250 mL conical flask, 5 mL of each Fehling solution A and B were pipetted and heated to boiling, then 48 mL of standard sugar was added which changed the colour of solution from blue to orange-brown. 2-3 drops of methylene blue indicator were added with continuous boiling and stirring. The titration was carried out until the colour changed to reddish brown due to cuprous oxide formation (endpoint).

$$V\ (blank) = V_f - V_i$$
$$= 41.8 - 0$$
$$= 41.8\ mL$$

From this step Fehling factor (strength of copper sulphate solution) was calculated:

$$\text{Fehling factor} = \text{titrated value} \times 0.001$$
$$= 41.8 \times 0.001$$
$$= 0.0418$$

- Sample titration

1 gm of the honey sample was accurately weighed in a 250 volumetric flask. It was diluted with 250 mL of distilled water and mixed well. In a conical flask, 5 mL of each Fehling solution A and Fehling solution B were added. From the burette around 12 mL of honey, the solution was added and then boiled (the solution colour was orange-brown). After adding 2-3 drops of methylene blue indicator with continuous boiling and stirring, the solution was titrated within 3 minutes until the colour changed from blue to reddish brown colour (endpoint)("https://law.resource.org/pub/in/bis/S06/is.4941.1994.pdf,"). Reducing sugar content was calculated as follows.

$$\text{Reducing sugar} = (250 \times 100 \times S) / (H \times M)$$

S = Fehling factor.
H = volume of honey solution required (burette reading).
M = mass of honey in gm.

3. RESULTS and DISCUSSION

A total of seven samples were collected that includes four marketed honey products collected from hypermarkets in Oman and 3 locally produced samples viz., Omani Sumer, Sidr, and Zah'r. The physicochemical parameters of these samples were evaluated and compared with the GCC standardization organization (GSO), International Honey Commission (IHC) limits, and the literature data.

3.1. Moisture Content

Moisture contents of the honey sample were measured by an Abbe refractometer. The honey moisture content depends on the methods of extraction, preservation, and storage. The moisture contents above the limits may cause microbial growth and further loss in taste and low shelf life. The percentage above 17% increases the chance of microbial growth however the moisture contents below the limit of 20% increase the shelf life of honey samples. The water contents may vary with the interaction of sugars present in the honey and low water contents prevents the microorganism attack as hyperosmotic honey will draw the water from the microorganism and kill them (Malika *et al*., 2005). The normal range of moisture content is between 13.7-18.8% for Sidr, 14.9-18.3% for Sumer, and 14-17.2% for Zah'r (Al-Farsi *et al*., 2018). None of the samples tested exceeds the limits as approved by the GSO. The results of the percentage moisture contents of the tested honey samples measured in triplicate are presented in Table 2.

Table 2. The moisture contents of the tested honey sample by Abbe refractometer.

S. No	Sample	Temperature (°C)	Refractive index	Refractive index (20°C)	Water content (g/100g)
1	AFS1	22.8	1.4936	1.4948	16.8
2	AFS2	23	1.4973	1.4986	15.2
3	AFS3	23.2	1.4973	1.4987	15.2
4	AFS4	22.7	1.4973	1.4985	15.2
5	ASF5	23.5	1.4970	1.4985	15.2
6	ASF6	22.8	1.4973	1.4985	15.2
7	ASF7	22.6	1.4973	1.4984	15.4

AFS1; Pure Australian honey, AFS2; Natural bee honey Saudi Arabia, ASF3; Pure bee honey Dubai, ASF4; Sumer (*Acacia tortilis*), ASF5; Sidr (Ziziphus), ASF6; Zah'r (Flower), ASF7; Australian origin honey.

3.2. Acidity, pH, and % Insoluble Matter

The pH corresponds to the quality of the honey sample (stability, texture, flavor, and shelf life), and an increase in acidity may affect the growth of mould and yeast in the honey sample. The pH ranges for the Sidr, Sumer and the Zah'r honey is 4.71-7.51, 4.12-4.90, and 3.46-4.79 (Al-Farsi *et al*., 2018). The minimum and maximum pH range reported by White was between 3.5 to 4.5 (WHITE, 1975). The presence of gluconic acid in all honey is due to the oxidation of glucose by the glucose oxidase activity added by honeybees during ripening. The acidification fastens the healing process by releasing oxygen from haemoglobin and makes a less favourable environment for the destructive proteases (Molan & Rhodes, 2015). The increase in the acidity of the sample might be due to inappropriate storage (duration and temperature) and processing conditions. The accepted range for free acidity according to GSO should not be more than 50 millimoles/kg (Al-Farsi *et al*., 2018). The results of the pH showed that all the honey tested was acidic and in conformity with results carried out by another research group 3.40-6.10 (El Sohaimy *et al*., 2015). The acidity values are within the limit for most of the tested samples. The sample AFS1 has a marginal increase in acidity with 51 millimoles/kg. The acidity results for the Sumer (AFS4) and Zah'r (ASF6) showed increased acidity with 78 and 61 mM/kg. The

acidity adds to the flavours and protection against the microorganism. The acidity is due to the contents of gluconic acid and glucolactone. The insoluble matter expressed for Sidr, Sumer, and Zah'r was 0.45, 0.4, and 0.35% below the maximum limit of GSO (0.5%). The insoluble matter present in other honey samples is above the limits of GSO (0.1%)(Al-Farsi *et al*., 2018). The results of the pH, acidity, and the % insoluble matter measure three times are shown in Table 3.

Table 3. Acidity in (mM/kg), pH and % insoluble matter (g/100g) of the honey samples.

S. No	Sample	pH	mL of 0.1 M NaOH after titration	Acidity (mM/kg)	weight of honey after filtration (g)	% Insoluble matter (g/100 g)
1	AFS1	3.69	5.1	51	0.06	0.3
2	AFS2	4.25	2.7	27	0.05	0.25
3	AFS3	4.32	1.3	13	0.06	0.3
4	AFS4	5.12	7.8	78	0.08	0.4
5	ASF5	4.80	1.9	19	0.09	0.45
6	ASF6	3.96	6.1	61	0.07	0.35
7	ASF7	4.68	0.8	8	0.06	0.3

AFS1; Pure Australian honey, AFS2; Natural bee honey Saudi Arabia, ASF3; Pure bee honey Dubai, ASF4; Sumer (*Acacia tortilis*), ASF5; Sidr (Ziziphus), ASF6; Zah'r (Flower), ASF7; Australian origin honey.

3.3. Proline Content

The proline is the main amino acid and its content directly indicates the honey ripeness. It measures the quality and antioxidant activity of the honey (Truzzi *et al*., 2014). The proline contents should not be less than 25mg/100g (Puścion-Jakubik *et al*., 2020). Although there is no limit for the proline contents in the GSO standard, however, a proline content of less than 180 mg/kg is considered unripe or adulterated by sugar addition in Germany (Al-Farsi *et al*., 2018). Our measured proline content in the tested honey samples showed that none of the samples has lower proline contents as prescribed by different countries. The proline contents of the measured honey samples measured in triplicate are shown in Table 4.

Table 4. The proline contents (mg/kg) of the tested honey sample.

S. No	Sample	Abs of sample	Abs of proline (Average)	Proline contents (mg/kg)
1	AFS1	0.176		370.52
2	AFS2	0.160		336.84
3	AFS3	0.121		254.73
4	AFS4	0.299	0.152	629.47
5	ASF5	0.169		355.78
6	ASF6	0.229		482.10
7	ASF7	0.118		248.42

AFS1; Pure Australian honey, AFS2; Natural bee honey Saudi Arabia, ASF3; Pure bee honey Dubai, ASF4; Sumer (*Acacia tortilis*), ASF5; Sidr (Ziziphus), ASF6; Zah'r (Flower), ASF7; Australian origin honey.

3.4. Hydroxy Methyl Furfural (HMF) Level

The hydroxy methyl furfural formation in the honey samples takes place at high temperatures in acidic conditions. The HMF is a major intermediate product of the Maillard reaction and is a browning reaction between sugars and free amino acids on prolonged storage and heating conditions (Chou *et al*., 2020). According to the reports, high HMF values may alter the flavour

and colour of the honey samples due to caramelization and degradation of honey samples. The average HMF value for the honey samples for sale should not exceed 40 mg/kg and may be affected by pH, acidity, moisture, and storage. The EU standard and Codex Alimentarius have also fixed the maximum HMF value for honey should not exceed 40 mg/kg (tropic ambient temperature honey should not be more than 80 mg/kg) (Chou *et al*., 2020). The GSO limits for the HMF contents are not more than 80 mg/kg. Our results for the HMF values for the tested honey samples are within the recommended range according to the GSO limit (80 mg/kg). The HMF contents are measured in triplicate and are represented in Table 5.

Table 5. The hydroxy methyl furfural (HMF) contents present in the honey samples.

S. No	Sample	Absorbance at 336 nm	Absorbance at 284 nm	HMF (mg/kg)
1	AFS1	0.306	0.492	41.766
2	AFS2	0.441	0.465	8.982
3	AFS3	0.248	0.401	34.35
4	AFS4	0.377	0.486	24.47
5	ASF5	0.383	0.477	21.10
6	ASF6	0.235	0.486	18.78
7	ASF7	0.315	0.517	45.35

AFS1; Pure Australian honey, AFS2; Natural bee honey Saudi Arabia, ASF3; Pure bee honey Dubai, ASF4; Sumer (*Acacia tortilis*), ASF5; Sidr (Ziziphus), ASF6; Zah'r (Flower), ASF7; Australian origin honey.

3.5. Total Reducing Sugars

According to the GSO honey standard, the total reduced sugars contents should be above 45%. Among the seven tested samples, four of the samples were below the limit in terms of total reducing sugar. The sugar levels in the honey influence the efficacy of the honey and may be affected by long storage during processing. The factors such as moisture levels, area of the honey harvested, and harvest time affect the sugar contents. The high glucose ratio allows honey to crystallize whereas other sugars present inhibit it (Ayubi, 2017). The total reducing sugar in the honey samples is measured in triplicate and represented in Table 6.

Table 6. Determination of reducing sugars in the honey samples.

S. No.	Sample	Initial reading	Final reading	Difference	Reducing sugars g/100 g
1	AFS1	0 mL	29.5	29.5	35.42
2	AFS2	0 mL	15.1	15.1	69.21
3	AFS3	0 mL	29.3	29.3	35.67
4	AFS4	0 mL	33.7	33.7	31.01
5	ASF5	0 mL	21.7	21.7	48.16
6	ASF6	0 mL	24.2	24.2	43.18
7	ASF7	0 mL	18.9	18.9	55.29

AFS1; Pure Australian honey, AFS2; Natural bee honey Saudi Arabia, ASF3; Pure bee honey Dubai, ASF4; Sumer (*Acacia tortilis*), ASF5; Sidr (Ziziphus), ASF6; Zah'r (Flower), ASF7; Australian origin honey.

4. CONCLUSION

A total of seven honey samples (three Omani honey and four marketed honey) were collected from local markets in Oman. The Omani honey includes Sumer, Sidr, and Zah'r types. All seven honey samples confirm the test limits (moisture, pH, proline, and HMF) approved either

by International or the GSO standards. The acidity of the three samples was found to be above the limits that may affect the growth of moulds and yeast. The insoluble matter expressed for Sidr, Sumer, and Zah'r was below the maximum limit of the GSO (0.5%) while the insoluble matter present in other honey samples was above the limits of the GSO (0.1%). The total reducing sugar of four samples was below the limit which indicates adulteration according to the GSO. The adulteration in terms of microbial or non-microbial, heavy metal, pesticides, and antibiotics contamination of the honey products may cause health hazards. Thus, the correct physicochemical analysis supports the originality and safety of honey products. In our study, most of the tested parameters of honey samples were within the limits still monitoring is required to improve processing and storage conditions for better honey quality to conform with the international standard limits in terms of both quality and quantity.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). **Ethics Committee Number**: EBS/18/2021-2022, Ethics and Biosafety Committee, College of Pharmacy, National University of Science and Technology, Azaiba, Muscat, Sultanate of Oman.

Authorship Contribution Statement

Al Zahraa Mohammed Said Al Hadhrami: Investigation; Methodology. **Fatema Rashid Abdullah Al Mazrooei:** Investigation; Methodology. **Sheikha Mohammed Ali Al Mamari:** Investigation; Methodology. **Sakina Habib Juma AL Humaid:** analyzed and interpreted data. **Shah Alam Khan:** Formal analysis, supervision. **Md Jawaid Akhtar:** Conceptualization, Data curation. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Published at https://dergipark.org.tr/en/pub/ijsm **Review Article**

Review on antibiotics residues and their extraction and detection methods in highly consumed foodstuffs

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Abstract: Antibiotics have been widely used in the food industry, and their utilization has increased tremendously. Foodstuffs sometimes comprise excessive amounts of antibiotic residues due to a lack of awareness and misuse of these valuable drugs. The misuse of antibiotics in foods has led to the growth of bacterial resistance. Over the past century, the increasing use and abuse of antibiotics in food animals have directed to the prevalent transmission of bacterial and genetic resistance between animals and humans. Antibiotic residue from foods is considered a significant contaminant that threatens human health worldwide. Awareness and training on the application of antibiotics among farmers and drug sellers can rationalize the use of antibiotics in food animals. The Government of Oman should create and firmly implement application guidelines to regulate the use and prevent the misuse of antibiotics in foodstuffs sectors. This review aims to explore the current status of antibiotic residue in foodstuffs, and their detection, separation, and identification technologies in use. The review also highlights alternative ways to fight bacterial resistance.

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1. INTRODUCTION

Since their discovery during the 1930s, antibiotics have been used to treat and prevent infections and preserve food, reflecting their dramatic impact on the fields of medicine and the food industry. The rapid application of antibiotics in the food process was initially known as progress in the food industry to be discovered later as a serious threat to human health.

Inappropriate antibiotic use in food animals can generate a potential risk for human or consumer health. Recently this risk increased because of the spread of these antibiotics for treatment, prevention, or growth promotion in almost all livestock (Van *et al*, 2019, FDA 2017). This issue was studied and discussed repeatedly and generated a lot of debate, but no action

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was taken to mitigate the possibility of potential transfer of resistance from animals to humans as pathogenic organisms (Diarrassouba *et al*, 2007). The extensive use of antibiotics was evident. For example, in American farms, nearly all dairy cows receive prophylactic beta-lactam treatment post-lactation as a preventive measure. The extensive use of antibiotics was evident. For example, in American farms, nearly all dairy cows receive prophylactic beta-lactam treatment post-lactation as a preventive measure. Ten percent of healthy calves are treated with antibiotics to manage anticipated outbreaks of respiratory disease.

Forty-two percent of kinds of beef received macrolide drugs to prevent liver disease that negatively impacts their growth. Similarly, antibiotics are added to >80% of growing swine food to prevent infections and promote swine growth (Department of Agriculture -U.S., 2007 & 1999). A veterinary prescription is required for antibiotic use in livestock; however, lay farm men often make and administer individual treatment decisions (Raymond *et al*, 2006).

Different types of antibiotics, such as natural, synthetic, or semi-synthetic antibiotics, are available in the markets and are used for treating or preventing infections in humans and animals (Kaza *et al.,* 2023; Wu *et al.,* 2018). The development and uses of antibiotics are one of restorative practices in the healthcare sector. Antibiotics are medications that are used to treat infections caused by bacteria. They work by either killing the bacteria or inhibiting their growth. Doctors often prescribe antibiotics to treat bacterial infections such as pneumonia, bronchitis, urinary tract infections, and skin infections (Tripathi & Tripathi, 2017; Wang *et al.,* 2020; Kumar & Pal, 2018; Chen *et al.,*2019; Phillips, 2003; Kneebone *et al.,* 2010; Jammoul & Darra, 2019; Farouk *et al.,* 2015). Antibiotics can be taken orally in the form of pills or liquids, or they can be administered intravenously through a vein. It is essential to take antibiotics exactly as prescribed by a doctor and finish the entire course of treatment, even if the symptoms of the infection have improved. This is because not completing the entire course of treatment can lead to the development of antibiotic-resistant bacteria, which can be more challenging to treat (Larsson, 2014; Majdinasab *et al.,* 2020; Al Salah *et al.,* 2019; Anand *et al.,* 2012).

The misuse or overuse of antibiotics during the production of food has directed to create antibiotic-resistant bacteria and antibiotic-resistance genes that could accumulate in foodstuffs and humans (Bai *et al.,* 2021; Karkman *et al.,* 2018; Jiang *et al.,* 2018; Rizzo *et al.,* 2014; Chowdhury *et al.,* 2021; Zhang *et al.,* 2020). Evidence is available related to antibiotic resistance in humans due to the vast application and misuse of antibiotics during the production of foodstuffs (Nguyen *et al.,* 2019). People widely use antibiotics to treat various infectious diseases and increase the production of foods. However, many users of antibiotics are not familiar with the proper use and dose of antibiotics for producing foodstuffs. Oman is not an agricultural country, but still, they produce some agricultural products. Furthermore, Oman has a huge number of chicken farms, and they produce chicken meat. The local farms cover about 60% of the protein requirement (Al-Bahry *et al.,* 2009). Farmers and chicken farm owners are using excessive amounts of antibiotics to produce huge crops and poultry. However, no study has been conducted in Oman on the status of antibiotic use and misuse in agriculture. This current review will provide a shade on the adverse effects of antibiotics use in food animals for future research and the correct application of antibacterial agents to reduce the harmful effects of antibiotic residue in foodstuffs.

2. TYPES OF ANTIBIOTICS

Different types of antibiotics are available all over the globe; they are classified based on their mechanism of action and activity. Some common types of antibiotics include penicillin, cephalosporins, tetracyclines, and macrolides (Al-Bahry *et al.,* 2912; Al-Bahry *et al.,* 2019; Hamilton-Miller, 1973; Heesemann, 1993; Henry, 1943; Holten & Onusko, 2000; Kahne *et al.,* 2005; Kang & Park, 2005; Livermore *et al.,*2011; Sykes & Bonner, 1985; Sykes *et al.,* 2015).

Each type of antibiotic is effective against certain types of bacteria, so a prescriber needs to determine by the culture test to select the appropriate antibiotic for a particular infection. Here is a brief overview of some of the main types of antibiotics (Al-Bahry *et al.,* 2912; Al-Bahry *et al.,* 2019; Hamilton-Miller*,* 1973; Heesemann, 1993; Henry, 1943; Holten & Onusko, 2000; Kahne *et al.,* 2005; Kang & Park, 2005; Livermore *et al.,*2011; Sykes & Bonner, 1985; Sykes *et al.,* 2015):

1. Penicillins: including drugs such as penicillin and amoxicillin and work by inhibiting the synthesis bacterial cell wall. They often treat respiratory tract, urinary tract, and skin infections.
2. Cephalosporins: This group of antibiotics, including drugs such as cephalexin and cefuroxime, inhibits the synthesis of the bacterial cell wall and can inhibit the synthesis of other bacterial cell components. They often treat respiratory, urinary tract, and skin infections.
3. Tetracyclines: including drugs such as doxycycline and minocycline, which inhibit the synthesis of proteins in bacterial cells. They often treat acne, respiratory infections, and sexually transmitted infections.
4. Macrolides: This group of antibiotics includes erythromycin and azithromycin. They inhibit the synthesis of proteins in bacterial cells and are often used to treat respiratory and skin infections.
5. Quinolones: Quinolones, including ciprofloxacin and levofloxacin, inhibit the synthesis of DNA in bacterial cells and are used to treat various infections, including respiratory tract infections, urinary tract infections, and sexually transmitted infections.
6. Aminoglycosides: include drugs such as gentamicin and amikacin and work by inhibiting the synthesis of proteins in bacterial cells. They are often used to treat serious infections such as pneumonia and sepsis.
7. Sulfonamides: including drugs such as sulfamethoxazole. They inhibit folic acid synthesis in bacterial cells and are often used to treat urinary tract infections and skin infections.

3. USE OF ANTIBIOTICS

Antibiotics are medications used to treat infections caused by bacteria. They work by either killing the bacteria or inhibiting their growth and are often prescribed by doctors to treat a wide range of bacterial infections. Antibiotics are available in various forms, including oral pills and liquids, intravenous solutions, and creams or ointments for topical use (Gothwal & Shashidhar, 2015; Liu *et al.,*2017; Abdel-Shafy & Mansour, 2018).

Antibiotics are usually prescribed based on the type of infection being treated and the susceptibility of the bacteria causing the infection to different types of antibiotics. In some cases, a doctor may prescribe a broad-spectrum antibiotic, which is effective against a wide range of bacteria, while in other cases, a narrow-spectrum antibiotic, which is effective against a specific type of bacteria, may be more appropriate (Menkem *et al.,* 2019; Gothwal & Shashidhar, 2015; Liu *et al.,*2017; Abdel-Shafy & Mansour, 2018).

It is worth noting that antibiotics are only effective against bacterial infections and cannot be used to treat infections caused by viruses, such as the common cold or influenza (Joshi & Ahmed, 2016). Using antibiotics unnecessarily or inappropriately can contribute to the development of antibiotic resistance, which occurs when bacteria become resistant to the effects of an antibiotic. This can lead to the spread of antibiotic-resistant bacteria, which can be more difficult to treat and require more potent or specialized antibiotics. It is important to use antibiotics only when necessary and to follow proper prescribing guidelines to help reduce the risk of antibiotic resistance (Gothwal & Shashidhar, 2015; Liu *et al.,*2017; Abdel-Shafy & Mansour, 2018).

In addition to being prescribed by doctors to treat specific infections, antibiotics may also be used in other settings to prevent infections. For example, antibiotics may be given to people who are undergoing surgery or other medical procedures to prevent infections. They may also be given to people who have compromised immune systems, such as cancer patients or HIV/AIDS patients, to prevent infections from occurring (Al-Bahry *et al.,* 2012; Livermore *et al*., 2011).

It is important to be aware of the potential side effects of antibiotics, which can vary depending on the specific drug being taken. Common side effects of antibiotics include nausea, diarrhea, and allergic reactions. Some antibiotics can also cause changes in the normal bacterial flora of the body, which can lead to the development of yeast infections or other types of infections. In rare cases, antibiotics can cause more serious side effects such as liver or kidney damage or blood disorders (Jammoul & Darra, 2019; Farouk *et al.,* 2015; Larsson, 2014; Majdinasab *et al.,* 2020; Al Salah *et al.,* 2019; Anand *et al.,* 2012). If a patient experiences any severe side effects while taking an antibiotic, it is important to contact the doctor as soon as possible.

Overall, antibiotics are essential medications that can be used to effectively treat bacterial infections and prevent infections from occurring in certain situations. However, using them responsibly and only when necessary is important to help reduce the risk of antibiotic resistance. It is also important to be aware of the potential side effects of antibiotics and to follow the healthcare provider's instructions to ensure the safe and effective use of these drugs.

4. SOURCES OF ANTIBIOTICS

1. Several different sources have been used for the production of antibiotics. Some antibiotics are produced by bacteria, while others are produced by fungi (Vannuffel & Cocito, 1996; Walsh, 2003; Sanchez *et al.,* 2004; Levy, 2007; Chadwick & Goode, 1997; McEwen & Fedorka-Cray, 2002). Here is a brief overview of some of the primary sources of antibiotics: Bacteria: Many antibiotics are produced by bacteria as a means of protecting themselves against other bacteria. These antibiotics are often extracted from soil bacteria or from bacteria that live on the bodies of animals. Examples of antibiotics produced by bacteria include penicillins, cephalosporins, and tetracyclines (Levy, 2007; Chadwick & Goode, 1997; McEwen & Fedorka-Cray, 2002).

2. Fungi: Some antibiotics are produced by fungi as a means of protecting themselves against other fungi or bacteria. These antibiotics are often extracted from soil fungi or from fungi that live on the bodies of animals. Examples of antibiotics produced by fungi include griseofulvin and amphotericin B (Levy, 2007; Chadwick & Goode, 1997; McEwen & Fedorka-Cray, 2002).

3. Chemical synthesis: Some antibiotics, such as sulfonamides and quinolones, are produced through chemical synthesis rather than being extracted from natural sources such as levofloxacin, gatifloxacin, moxifloxacin, and sparfloxacin.

5. EXTRACTION OF ANTIBIOTICS

There are several methods available for the extraction of antibiotic residue the from foodstuffs The extraction methods depend on the types of antibiotics. Therefore, the extraction methods are varied based on the classification of antibiotics. All the methods are complex, and they have multiple steps to extract the desired antibiotics. The simplest and most popular methods for extraction of antibiotics from foodstuffs are given as follows:

5.1. Method 1

The food samples are pasted by using a kitchen blender machine. The paste sample (2 gm) is taken into a beaker (50 ml) and a buffer-EDTA solution is added (McEwen & Fedorka-Cray,

2002). The mixture is mixed using a homogenizer, transferred into a centrifuge tube (10 ml), and centrifuged at 3000 rpm for 1 minute. The supernatant part is transferred into another tube and evaporated to 1 ml. The concentrated sample is passed through the solid phase extraction column containing activated silica. An eluent organic solvent is used to clean the antibiotics and concentrate it using a rotary evaporator. The concentrated solution is analysed by using HPLC method. Methanol, acetonitrile, and formic acid are commonly used as mobile phases for the isolation and separation of various antibiotics. Most scientists use methanol and acetonitrile worldwide as the mobile phase because the solvent is affordable and available for enhancing the solubility and the analysis of antibiotics (Witte, 1998; Xu *et al.,* 2020; Li *et al.,* 2020).

5.2. Method 2

The foodstuffs samples (2 gm) are pasted and homogenized by the vertex machine for a few minutes. The sample is transferred into a beaker and added nitric acid and aqueous solution and the mixture is shaken vigorously. Then the sample is centrifuged at 4000 rpm for 10 minutes. After centrifugation, the sample is passed through the filter paper, and the filtrate is evaporated by as usual method until the concentration was 1 ml. The concentrated sample is analyzed by chromatographic method (Betina, 1993).

5.3. Method 3

The collected foodstuffs sample (2 gm) is prepared as a paste, taken into a beaker, and treated with acetic acid. The mixture is well mixed for a few minutes, and the sample is centrifuged at a specific rpm for 5 minutes. The supernatant liquid is filtered using filter paper and evaporated until 1 ml (Senyuva *etal.,* 2000; Markina *et al.,* 2020).

6. DETECTION OF ANTIBIOTICS

The literature shows that various types of chromatography, such as paper chromatography, thin-layer chromatography, and ion exchange chromatography, are used as basic tools for isolating, separating, and characterizing antibiotics from the foodstuffs. Due to the improvement of technology, recently High-Performance Liquid Chromatography (HPLC), Liquid Chromatography-Mass Spectrometry (LC-MS), and Ultra-High Performance Liquid Chromatography (UHPLC) are used for the separation, characterization, and quantification of various antibiotics in foodstuffs (McEwen & Fedorka-Cray, 2002; Witte, 1998; Xu *et al.,* 2020; Li *et al.,* 2020). Liquid chromatography-mass spectrometry (GC-MS) is a well-established method for detecting and quantifying various antibiotics in biological and animal samples (McEwen & Fedorka-Cray, 2002; Witte, 1998). However, all the mentioned methods are too expensive for routine analysis. Therefore, for the analysis of antibiotics currently, L.C. system is used coupled with different detectors such as U.V., diode array, and fluorescence detector. They are highly recommended as alternatives (Senyuva *etal.,* 2000; Markina *et al.,* 2020; McEwen & Fedorka-Cray, 2002; Witte, 1998; Xu *et al.,* 2020; Li *et al.,* 2020).

7. CONCLUSIONS

The improper use of antibiotics in the Agri-foods sector can result in antibiotic residues in food products, and these antibiotic residues can adversely affect human health. Several analytical methods were developed globally for detecting, isolating, and quantifying antibiotic residues in foodstuffs. LC-MS is a widely accepted technique for detecting and quantifying antibiotic residues in foodstuffs. Although, the LC-MS technique is highly sensitive, it is expensive and not affordable for the small-scale analysis of residues in foods. Therefore, scientists are always searching for comparatively cheap and available alternatives. As an alternative, scientists are currently using L.C. system coupled with sensitive and powerful detectors such as U.V., diode array, and fluorescence detector to analyse foodstuff antibiotics residues.

Several challenges must be considered when using antibiotics, including the increasing problem of antibiotic resistance, the potential for contamination during the production process, and the cost and accessibility of these medications. It is important to use antibiotics responsibly and only when necessary to help reduce the risk of antibiotic resistance and to ensure that these medications are accessible and affordable to those who need them. Overall, antibiotics are essential tools in treating bacterial infections, however we must use them responsibly to help preserve their effectiveness for the future.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Mohammad Amzad Hossain, Ahmed Abu Sham and Salem Said Jaroof Al-Touby: Conceptualized the Review articles. **Ali Attia Abedlnaeem Attia Salem, Waleed Khalid Hilal Al Rajhi**: Draft preparation. **Mohammad Amzad Hossain, Salem Said Jaroof Al-Touby**: involved in the drafting and edition. Drafts were critically discussed and revised by all authors.

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Biosynthesis of ZnO nanoparticles using *Laurus nobilis* leaf extract and investigation of antiproliferative and antibacterial activity potential

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Abstract: Nanotechnology has recently emerged as an essential field of study in modern materials science. The green synthesis of nanoparticles using plant extracts is of great interest because it is cost-effective, eco-friendly, and suitable for large-scale production. The study highlights the synthesis of ZnO nanoparticles (ZnO NPs) using *Laurus nobilis* (*L. nobilis*) leaf extract and their characterization and biological activities for potential applications in the biomedical field. ZnO NPs were synthesized using *Laurus nobilis* leaf extract. The synthesized ZnO NPs were characterized by UV-Vis spectroscopy, TEM, XRD, and FT-IR. According to TEM and XRD diffraction analysis, with a mean particle size of 16 ± 5 nm, it was found that the synthesized ZnO NPs contain a hexagonal wurtzite structure. ZnO NPs have antibacterial activity against Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Escherichia coli* (*E. coli*). The antiproliferative activity of ZnO NPs was tested against the human colon cancer cell line and mouse normal fibroblast cell line using MTT assay in vitro. The results show that the prepared nanoparticles had antiproliferative in screened incubation time and concentrations.

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1. INTRODUCTION

ZnO NPs, due to their superior properties, namely large binding energy, wide band gap, and high piezoelectricity, have many applications such as medical imaging (Lim *et al*., 2012; Senthilkumar *et al*., 2009), nanocomposites (Bhattacharya & Samanta, 2016), drug delivery (Yuan *et al*., 2010), and hyperthermia of tumors (Sharma *et al*., 2022). There are numerous methods to synthesize ZnO NPs, including mechanical grinding, chemical reduction, laser cutting, photoreduction, and the green approach (Agarwal *et al*., 2017; Ghimire *et al*., 2022; Wirunchit *et al*., 2021). Among them; the green approach is of great interest to reduce metal salts to nanoparticles because it is inexpensive, non-toxic, and environmentally friendly (Jadoun *et al*., 2021). There are many studies in the literature on the synthesis of ZnO NPs using different plant extracts such as *Nephelium lappaceumL.* (Karnan & Selvakumar, 2016) *Mangifera indica* (Rajeshkumar *et al*., 2018), *Suaeda aegyptiaca* (Rajabi *et al*., 2017), *Calotropis gigantea*

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(Vidya *et al*., 2013), *Aspalathus linearis* (Diallo *et al*., 2015), and *Syzygium Cumini* (Sadiq *et al*., 2021). Synthesis of nanoparticles from plant extracts is a bottom-up approach using metal salts as precursors (Hoda *et al*., 2021).

L. nobilis is an evergreen, aromatic plant of the Lauraceae family that grows in temperate and tropical areas of the world, including Türkiye, Spain, Morocco, Greece, Portugal, Mexico, and other Mediterranean nations (Alejo-Armijo *et al*., 2017). The leaves of this plant are used as a spice flavoring in foods. In addition, leaf extracts are used for fungal and antimicrobial infections, and to treat burping, bloating, and gastrointestinal issues (A. Sharma *et al*., 2012). *L. nobilis* leaf extract contains ingredients such as sugars, kaempferol glycosides, sesquiterpene lactones, megastigme glycosides, organic acids, (+)-catechin, (+)-gallocatechin, (+)-epigallocatechin, (-)-epicatechin, and procyanidins (Kaurinovic *et al*., 2010). Thanks to the components it contains, the plant can form and stabilize nanoparticles by reducing metallic ions (Rai *et al*., 2009).

Cancer, which is the primary cause of death worldwide, is one of the health problems that most concern and affect society. In 2020, colorectal cancer was the third cause of cancer-related death in the United States (Siegel *et al*., 2020). Depending on the type of cancer, its stage, and the patient's health, several treatment methods, including surgery, radiotherapy, and chemotherapy, are used (Debela *et al*., 2021). However, these treatment methods have disadvantages such as selectivity, multi-drug resistance, and adverse side effects (Mondal *et al*., 2014). Recently, nanoparticles' small size and greater atomic content on their surface allow them to interact with biomolecules both inside and outside body cells, enabling them to be used in medicine to treat cancer as drug delivery systems and diagnostic tools (Mundekkad & Cho, 2022; Zabielska-Koczywąs & Lechowski, 2017).

In this study, *L. nobilis* leaf extract was used to synthesize ZnO NPs. The synthesized ZnO NPs were characterized by UV-Vis spectroscopy, XRD, FTIR, and TEM. *S. aureus* and *E. coli* strains were utilized to examine the antibacterial activity of ZnO NPs. In addition, the Minimum Inhibitory Concentration (MIC)-Minimum Bactericidal Concentrations (MBC) of ZnO NPs were determined. The antiproliferative activity of ZnO NPs was tested against a human colon cancer cell line and a mouse normal fibroblast cell line using MTT assay in vitro.

2. MATERIAL and METHODS

2.1. Chemical Used

Zinc acetate dihydrate ($Zn(CH_3CO_2)_2.2H_2O$) and Sodium hydroxide (NaOH) used in this study were purchased from Merck Company. Deionized water was used throughout the experimental study. *L. nobilis* leaves were collected from the Antalya region and their type has been confirmed.

2.2. Preparation of Aqueous Leaf Extract

L. nobilis leaves were washed with tap water and then with deionized water to purify them from dust and foreign wastes and dried them by laying in the shade at room conditions. After the dried *L. nobilis* leaves were ground, the powder was sieved with a particle size of 0.630 – 1.00 mm to be used in the extract. 2.5 g of powdered *L. nobilis* leaves and 100 ml of deionized water were taken into a 250 ml flask and boiled under reflux for 5 min. Then, the extract was cooled to room temperature and filtered. The extract was centrifuged at 9500 rpm for 5 minutes to remove plant residues and impurities and kept at 4°C for further studies.

2.3. Synthesis of Nanoparticles

Zinc acetate dihydrate was used as a metal precursor for the synthesis of ZnO NPs. ZnO NPs were synthesized using *L. nobilis* leaf extract with minor modification, as previously demonstrated by Dönmez (Dönmez, 2021). 10 ml of *L. nobilis* leaf extract was added to 90 ml

of 0.02 M zinc acetate solution and stirred at room temperature using a magnet stirrer. Then, 1 M NaOH was added drop by drop to reach the pH of the mixture to 12 and the solution was stirred at room temperature for 2 hours. The resulting white mixture was centrifuged at 9500 rpm for 30 min. The precipitate was purified with deionized water and ethanol to remove impurities. Finally, obtained white precipitate was dried at 60 overnight and then calcined at 200°C for 3 hours.

2.4. Characterization of Synthesized Nanoparticles

ZnO NPs were characterized by UV-Vis spectroscopy, XRD, FTIR, and TEM techniques. UV-visible spectra of ZnO NPs were recorded in the 200 to 700 nm wavelength range using a JASCO V-770 UV/Vis spectrometer operating at a 1 nm resolution. X-ray diffractometer with Cu Ka radiation wavelength of 0.15406 nm investigated the phase structure and material identification of ZnO NPs. Spectrum was obtained using Panalytical Empyrian X-ray diffractometry at an angle of 2θ in the range of 10° to 80°. The morphologies and dimensions of ZnO NPs were investigated by Transmission Electron Microscopy (JEOL JEM-1010). The stability of synthesized ZnO NPs was measured by zeta potential analysis (Horiba Scientific).

2.5. Bacterial Strains and Determination of Minimum Inhibitory Concentration (MIC)-Minimum Bactericidal Concentration (MBC)

To investigate the antibacterial activity of ZnO NPs Gram-positive *S. aureus* ATCC 25923, and Gram-negative, *E. coli* ATCC 25922 strains were used. The bacteria strains were cultured in Mueller Hinton broth (MHB) (Merck, Germany) at 37˚C for 18-24 h with 120 rpm.

The detection of MIC of ZnO NPs was carried out using a microtitre broth dilution method. The bacterial suspension was adjusted with sterile saline to a concentration of 10^8 CFU ml^{-1}. A hundred µL of ZnO NPs were added to the wells containing 100 µL MHB medium and serially diluted two-fold. Five µL of bacterial suspension adjusted to 0.5 McFarland inoculated each well and plates were incubated at 37 ºC'de for 24 h. Following incubation, microplates were evaluated. The smallest concentration without growth was determined as the MIC value. MBC test was performed by plating the suspension from each well of microtiter plates into the MHA plate. The plates were incubated at 37◦C for 24 h. The lowest concentration with no visible growths on the MHA plate was taken as the MBC value.

2.6. Investigation of the Effects of ZnO NPs Against Colon Cancer Cell Line and Mouse Adipose Fibroblast Cell Line

The human colon adenocarcinoma epithelial cell line (DLD-1) (ATCC® CCL-221TM) was purchased from the American Type Culture Collection (ATCC, USA). Mouse adipose fibroblast cell line (L929) (ATCC® CCL-1TM) was donated by Associate Professor Cigdem Yucel from Erciyes University, Faculty of Pharmacy. DLD-1 and L929 cell lines were cultured in DMEM supplemented with 10% FBS and 1% glutamax. The cell seeding was done at 5 × 103 cells/well density into 96-well plates. The DLD-1 and L929 cells were exposed to ZnO NPs at 50, 25, 12.5, 6.25, and 3.125 µL/mL concentrations for 48 h. The 50 µL of MTT stock solution, which is prepared as 5 mg/mL in DMEM solution, was added to the plate wells and, were incubated for 3 h. Absorbance values were measured in an Elisa plate reader at 590 nm.

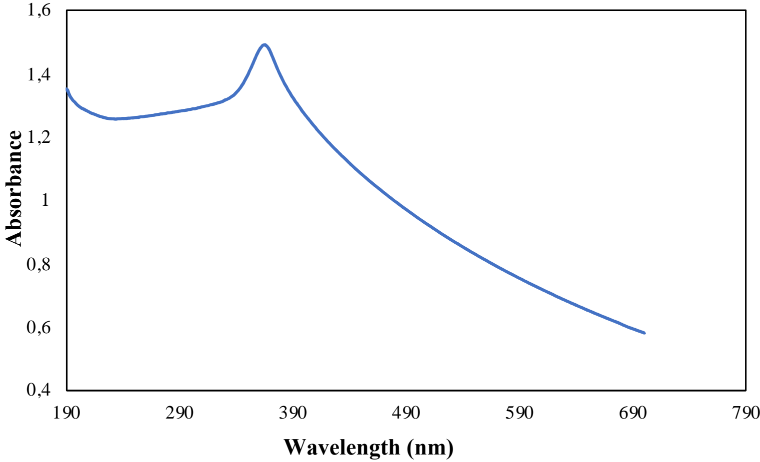
3. RESULTS and DISCUSSION

3.1. Characterization of ZnO NPs

In this study, the synthesis of ZnO NPs using *L. nobilis* leaf extract was successfully carried out. Firstly, UV-Vis analysis was performed to observe the formation of synthesized ZnO NPs. The UV-Vis spectrum of ZnO NPs is given in Figure 1 and the absorption peak at 372 nm belongs to the SPR absorption of it. The structure of the plasmon band and the wavelength

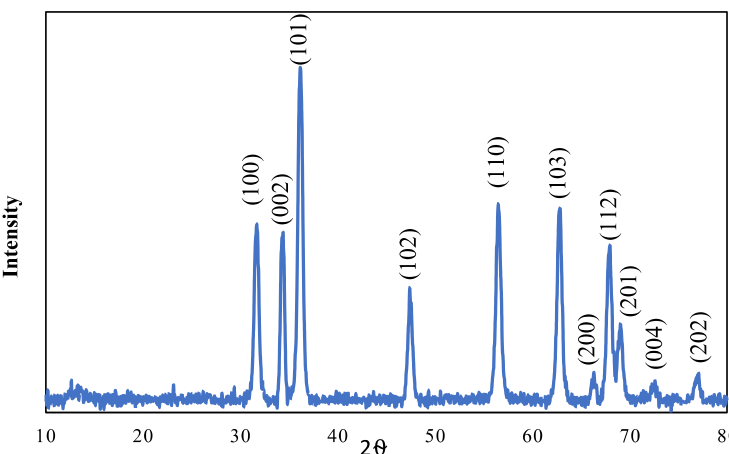
range in which it is observed vary according to the size distribution, average diameter, and shape of the nanoparticles (Link & El-Sayed, 1999). It is well known that the plasmon band gap in the UV-Vis Spectrum of ZnO NPs increases with decreasing particle size. For example, Hammad *et al*. (2010) investigated the change in particle size by exposing the synthesized ZnO NPs to different calcination temperatures and reported that there is a change in the plasmon band depending on the particle size in the UV-Vis spectrum of ZnO NPs of different sizes (Hammad *et al*., 2010).

Figure 1. UV-Vis spectrum of synthesized ZnO NPs.



The crystal structure of the synthesized ZnO NPs was determined by XRD diffraction analysis. The XRD patterns of the synthesized ZnO NPs are given in Figure 2. The XRD diffraction peaks of the synthesized ZnO NPs are in good agreement with the ZnO hexagonal wurtzite structure of the Joint Committee on Powder Diffraction Standards (JCPDS-36-1451). No characteristic diffraction peaks were observed in the XRD analysis except for ZnO, indicating that the ZnO NPs were free of unwanted impurities. Similar results have been reported in some studies in the literature (Al-Kordy *et al*., 2021; Khorsand Zak *et al*., 2011).

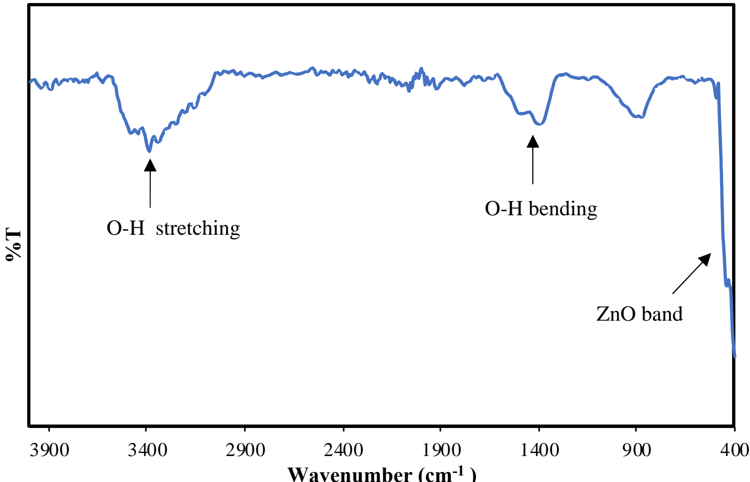
Figure 2. XRD pattern of synthesized ZnO NPs.



FTIR spectra of synthesized ZnO NPs were taken in the range of 400–4000 cm^{-1} as seen in Figure 3. The characteristic stretching peak of the ZnO bond is assigned a significant vibration band in the FTIR spectrum ranging from 400 cm^{-1} to 500 cm^{-1}. A large peak at 3388 cm^{-1} (stretching) and 1670 cm^{-1} (bending), is caused by ambient moisture, indicating the presence of hydroxyl residue (Nagaraju *et al*., 2017). The FTIR results are consistent with the results of

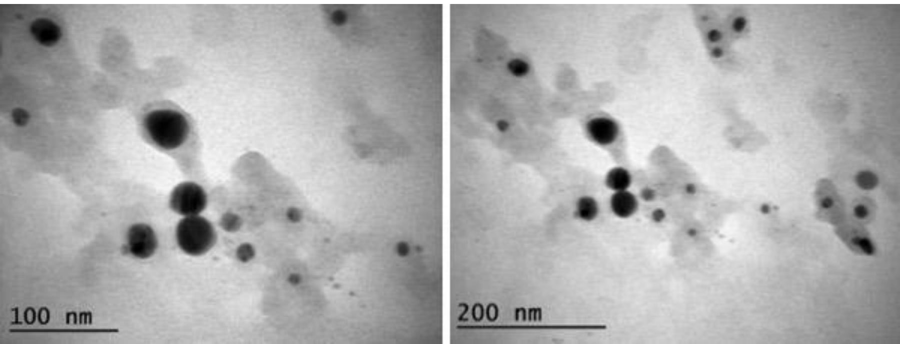
ZnO NPs which were synthesized from various plant extracts (Dobrucka & Długaszewska, 2016; Song & Yang, 2016).

Figure 3. FTIR spectra of synthesized ZnO NPs.



The TEM images of ZnO NPs synthesized using *L. nobilis* leaf extract, shown in Figure 4, can help us analyze the size and shape of the synthesized nanoparticles. According to the TEM images of ZnO NPs, it was observed that the nanoparticles ranged in size from 9 to 33 nm, with a mean diameter of 16 ± 5 nm. It also showed that the synthesized ZnO NPs were roughly spherical.

Figure 4. TEM images of synthesized ZnO NPs.

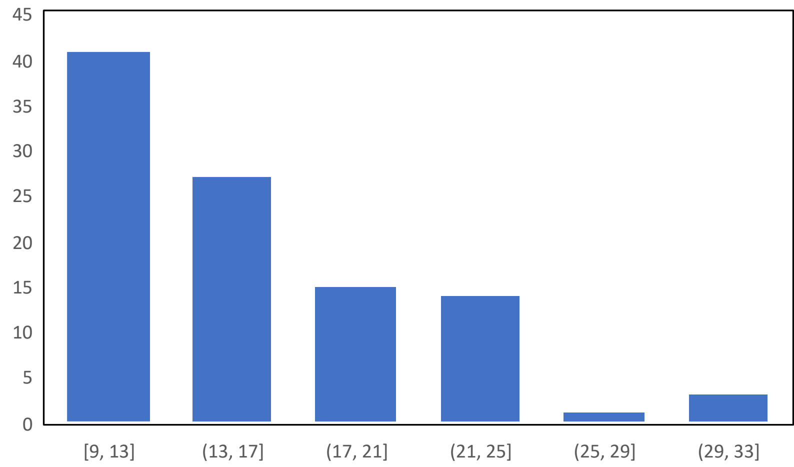


The zeta potential analysis confirms the stability of ZnO NPs synthesized using *L. nobilis* leaf extract. As seen in Figure 5, the zeta potential of the synthesized ZnO NPs is -25,2 mV. This result shows that the synthesized ZnO NPs are covered by negatively charged groups and are quite stable.

The size distributions of the synthesized ZnO NPs were examined by evaluating the TEM images with Adobe Photoshop 7. A total of 100 particles were counted and the size distribution plot for the corresponding particles is given in Figure 6. The results show that the size of the synthesized ZnO NPs is between 9 nm and 33 nm.

Figure 5. Zeta potential of synthesized ZnO NPs.

Figure 6. Particles size distribution of synthesized ZnO NPs.



3.2. Bacterial strains and Determination of MIC- MBC of ZnO NPs

Since ancient times, people have utilized the antibacterial properties of certain metals and their ions. Today, it is known that a variety of metals, including Ag, Al, Co, Cu, Fe, Ga, Mn, Ni, Pb, and Zn, have antibacterial properties (Gudkov *et al*., 2021). The capacity of metal ions to block enzymes, disrupt cell membranes, hinder the uptake of critically needed microelements by microorganisms, or induce DNA damage forms the basis of metals' antimicrobial activity (Turner, 2017). The MIC/ MBC results against the test bacteria are given in Table 1. The results showed that the ZnO NPs had antibacterial activity and the MBC and MIC of *S. aureus* and *E. coli* were 4,35 mg/mL and 2,175 mg/mL, respectively. The antibacterial activity of the *L. nobilis* leaf extact on bacteria was not observed at the studied concentration. The antibacterial effect of ZnO NPs has been demonstrated in many studies carried out so far (Anand *et al*., 2019; D. Sharma *et al*., 2010). The physical and morphological characteristics of nanoparticles and synthesis techniques change their antibacterial capabilities. For example, Upadhyaya *et al*. (2018) reported that hexagonal ZnO NPs synthesized using *Lawsonia inermis* extract showed higher antibacterial activity against S. aureus than rod-shaped ZnO NPs at 100, 200, and 500 µg/mL concentrations. They concluded that hexagonal ZnO NPs, due to their shape,

penetrate the cell membrane barrier more easily than rod ZnO NPs and cause cell death (Upadhyaya *et al*., 2018).

ZnO NPs show a higher antibacterial effect compared to bulk ZnO (Yamamoto, 2001). The factors that most affect the antibacterial activity of ZnO NPs are their small size and large surface/volume ratio. Akbar *et al*. (2017) reported that as the size of ZnO NPs decreases, the antibacterial effect increases (Akbar *et al*., 2017). The smaller nanoparticles effectively penetrate through the cell walls of the bacteria, causing membrane damage and ultimately leading to cell lysis. The high antibacterial effect of the synthesized ZnO NPs can be attributed to their small size and hexagonal structure.

Table 1. Antibacterial activity of ZnO NPs against microorganisms (MIC/MBC value).

Microorganisms	ZnO NPs
S. aureus ATCC 25923	2.175/1.08
E. coli ATCC 25922	2.175/4.35

3.3. Antiproliferative Activity Studies of ZnO NPs

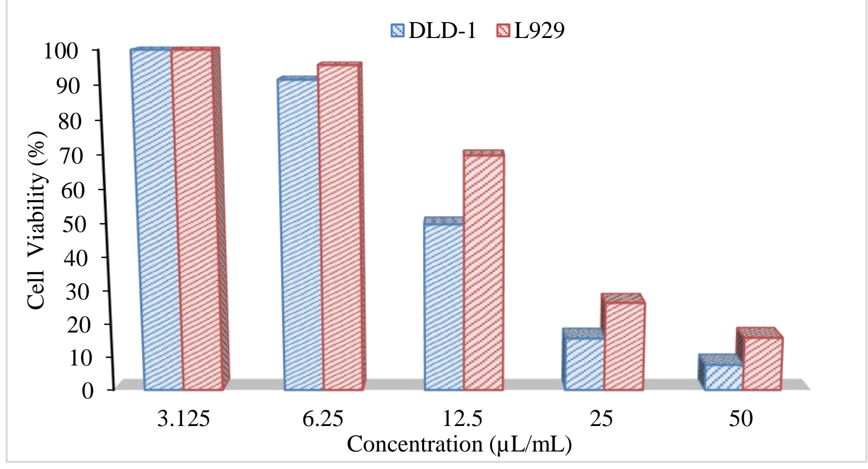
The synthesized ZnO NPs were tested in human colon cancer cell lines and mouse fibroblast cell lines *in vitro*. The results are given in Table 2.

Table 2. IC_{50} results of ZnO NPs in DLD-1 and L929 cell lines.

Compound	IC_{50} (µL/mL)	
	DLD-1	L929
ZnO NPs	13.85	20.08

The results show that ZnO NPs have obvious antiproliferative activity in the colon cancer cell line with the half maximal inhibitory concentration (IC_{50}) value of 13.85 µL/mL for 48 h. From the above IC_{50}) value, it is clearly seen that the synthesized ZnO NPs can inhibit the growth of colon cancer cells. It was also determined from the cell culture studies that the synthesized ZnO NPs showed selectivity on healthy cells (L929) and IC_{50} value was obtained as 20.08 µL/mL. The normal and cancer cell viability ratios depending on concentrations of ZnO NPs are given in Figure 7.

Figure 7. Changing cell viability rates depending on the concentrations of synthesized ZnO NPs.



It is seen that the synthesized ZnO NPs has an effect on cell viability depending on the concentration (Figure 7). At 3.125 µL/mL concentration, the ZnO NPs was found to be inactive on both healthy (L929) and cancerous (DLD-1) cells. In the colon cancer cell line, it is seen

that the cell viability rates interacting with the ZnO NPs decreased by 91.57%, 49.88%, 15.74%, 7.64% for 6.25, 12.5, 25 and 50 µL/mL, respectively, as the concentration increased. It is clearly seen that the synthesized ZnO NPs at 12.5 µM have selectivity on healthy cells and the viability rates were obtained as 49.88% and 69.93% for DLD-1 and L929, respectively. At 50 µL/mL of the ZnO NPs, the viability of healthy cells was almost twice that of cancer cells at 15.95%.

4. CONCLUSION

In this study, ZnO NPs were successfully synthesized from *L. nobilis* leaves extract via the green approach, which is an inexpensive, non-toxic, and eco-friendly method. The synthesized ZnO NPs were characterized by UV, TEM, XRD, and FT-IR. According to XRD diffraction analysis of ZnO NPs, it was observed that they have a hexagonal wurtzite structure. The size and shape of the ZnO NPs were determined using the TEM method. According to the TEM images of ZnO NPs, it was observed that the nanoparticles ranged in size from 9 to 33 nm, with a mean diameter of 16 ± 5 nm. To investigate the antibacterial effect of ZnO NPs *S. aureus*, and *E. coli* strains were used. The MIC/MBC results of ZnO NPs against test bacteria showed that they had antibacterial activity and MBC and MIC values of *S. aureus* and *E. coli* were 4.35 mg/mL and 2.175 mg/mL, respectively. Bacterial infectious illnesses are a severe public health issue that has gained international attention as a hazard to human health that also has implications for the economy and society. Therefore, new antibacterial agents are needed to combat infectious diseases. Although the antibacterial effect of nanoparticles on many microorganisms is seen as a promising approach, more research is required on it.

In addition, synthesized ZnO NPs were screened in DLD-1 and L929 cell lines. The nanoparticles were found to be effective in DLD-1. Furthermore, it has selectivity against normal cell lines. Synthesized ZnO NPs have the potential to be used in biomedical applications thanks to their antibacterial and anticancer activity.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Firdevs Mert Sivri, Cennet Çirrik, and **Aleyna Ezer:** Carried out the L. nobilis leaves extract and synthesis of ZnO NPs using L. nobilis leaf extract. **Ebru Önem:** Determined the antibacterial activity, and the Minimum Inhibitory Concentration (MIC)-Minimum Bactericidal Concentrations of ZnO NPs. **Senem Akkoç:** Tested the antiproliferative activity of ZnO NPs against a human colon cancer cell line and a mouse normal fibroblast cell line using the in vitro MTT assay. All authors wrote down the methods and discussion texts of the experiments they performed in the study. All authors reviewed the manuscript.

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Research Article

Investigation of antimicrobial and anticancer activity of extracts obtained following UV application to *Althaea officinalis* L. callus cultures

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Abstract: *Althaea officinalis* L., a plant of the Malvaceae family, is widely used in alternative medicine. The aim of this study is to cultivate the *Althaea officinalis* plant under *in vitro* conditions to create an appropriate callus regeneration protocol and investigate the antimicrobial and anticancer activities of methanol and ethyl acetate extracts of calli after UV-C application. Leaf, petiole, and root parts of *A. officinalis* plants germinated in a sterile environment were used as explant sources. Explants were cultured on MS medium containing different concentrations of 2,4-D (1, 2 mg/l) and BAP (0.25, 0.50, 0.75 mg/l). The most effective (100%) callus growth and callus weight (516.24±0.48 mg) was observed on petiole explants using MS medium containing 1 mg/l 2,4-D + 0.25 mg/l BAP. Calli obtained from leaf and petiole explants were exposed to UV-C treatment. Extractions of calli were carried out using methanol and ethyl acetate solutions. 1 mg/ml, 5 mg/ml, and 10 mg/ml solutions of methanol and ethyl acetate extracts were prepared and their antimicrobial activity on bacteria was investigated using the disc diffusion method for 7 different gram-positive and 9 different gram-negative bacteria. None of the three extract concentrations used had any antimicrobial activities. The anticancer activities of the extracts on SH-SY5Y human neuroblastoma cells were studied using the WST-1 viability kit. 1000, 500, 250, 125, and 62.5 µg/ml concentrations of ethyl acetate extracts of leaf and petiole calli had anticancer activity.

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1. INTRODUCTION

Althaea officinalis L. or Marshmallow is a perennial and herbaceous plant. *A. officinalis* is widely distributed in the Mediterranean, North Africa, France, the United Kingdom, the Balkans, Central Asia, and Russia. It can grow on seasides, salt marshes, and damp meadows (Altan, 2001). Since ancient times, medicinal and aromatic plants have been used for therapeutic purposes in folk medicine, but there was no extensive knowledge about the content of the plants. Plants are of great importance and use in the fields of chemistry, biology, pharmacy, medicine, and biotechnology (Bodeker *et al*., 2002, Al-Snafi, 2013). Marshmallow is used as a pain reliever against infections, a diuretic effect, a chest softener and protector, an immune system booster for persistent coughs, weight loss, softening of skin wounds, protective

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and wound healing, and relieving respiratory tract damage due to cough. The roots, leaves, and flowers of the plant are used for different medicinal purposes in many countries worldwide (Elmastas *et al*., 2004). It is used in the treating irritation of the mouth and throat mucosa, in the treatments of dry cough, mild gastritis, skin burns and insect bites, ulcers, abscesses, burns, and constipation In addition to these medicinal purposes, it has also been used in the food industry (Changizi *et al*., 2015). *A. officinalis*, also provides a natural and beautiful appearance to the hair and strengthens vision. It helps to regulate body weight by melting fats, increases performance during exercise, and relieves thirst (Diplock, 1998; Baytop, 1984).

It increases sexual performance and heals wounds on the skin. It removes wrinkles, freckles, and brown spots on the skin. It moisturises the skin. It is used in the treatment of haemorrhoids. While it has a calming effect, it eliminates the feeling of tiredness and exhaustion. It is effective in normalising blood pressure. It treats cough and bronchitis. It relieves muscle and headaches. It prevents insomnia by regulating the nervous system (Diplock, 1998). It allows the hardened liver to soften and renew itself and helps to stabilise blood glucose levels by regulating it. It helps in the treatment of digestive system and stomach ailments. It is effective in lowering high blood pressure and relieves restlessness.

It improves kidney activities. It is used to prevent and treat cancer, and it ensures the regeneration of the cell wall. It helps to remove uric acid and cholesterol from the body by dissolving them in water. It stimulates the secretory glands, encourages the release of hormones, helps the waste and toxic substances in the body become water-soluble and expelled, and accelerates blood circulation. It has a stimulating effect on metabolism. Moreover, it regulates the heartbeat and it cleans the blood (Baytop, 1984).

This study aims to investigate the antimicrobial and anticancer activities of methanol and ethyl acetate extracts of calli by using different types of explants from *in vitro* germinated *A. officinalis* as a source and creating a suitable callus regeneration protocol after UV-C application.

2. MATERIAL and METHODS

2. 1. Plant Material

The seeds of the *A. officinalis* used in this study were collected from its natural growing environment. The species was identified according to "The Flora of Turkey and East Aegean Islands" (Davis 1984).

2.2. Sterilization

The surface sterilisation of *A. officinalis* was carried out by using commercial bleach (ACE – Turkey, 5% NaOCl). The most suitable sterilisation protocol was developed by keeping the seeds in bleach for 10, 15, 20, 25, and 30 minutes. Following the sterilisation, the seeds were rinsed with sterile distilled water three times to remove the sodium hypochlorite traces on their surface.

2.3. Nutrient Environment and Culture Conditions

In this study, MS medium containing 3% sucrose and solidified with 0.35% gelrite was used to ensure the germination of seeds. The pH of the media was adjusted to 5.8 ± 0.2 using 1N NaOH and HCl. Leaf, petiole, and root parts of sterile *A. officinalis* plantlets grown by germination of sterile seeds were used as explant sources. Explants were cultured on MS nutrient medium containing 3% sucrose, solidified with 0.35% gelrite, pH adjusted to 5.8 ± 0.2 using 1N NaOH and HCl, amended with 2,4-D (1.2 mg/l) and BAP (0.25, 0.50, 0.75 mg/l). To ensure optimal conditions for calli development, the culture dishes were placed in an environmental cabinet and maintained at a constant temperature of 25±1 °C. The photoperiod consisted of 16 hours of light followed by 8 hours of darkness, with a light intensity of 500 µmol $m^{-2}s^{-1}$.

2.4. UV-C Application to Callus Cultures

Short-wavelength UV-C light was used in the study. UV-C light was applied for 15 minutes from a distance of 15 cm by opening the covers of the magenta vessels where calli were developed.

2.5. Obtaining Methanol and Ethyl Acetate Extracts from Calli

After the calli developed in the callus development medium, they were dried and powdered, 100 g of callus was taken, and 2000 ml of methanol or ethyl acetate was added to it and kept in a shaker incubator for 24 hours under normal conditions. At the end of the 24 hours, the macerated solution was passed through the filter paper, and the large particles were removed. Then the solutions were removed with the help of a rotary evaporator.

2.6. Antimicrobial Activity

The antimicrobial activities of the extracts were determined using the disk diffusion method. For this, the microorganisms used in the study were multiplied in a liquid nutrient medium (LB Broth, Germany) with a final concentration of 10^6. The final concentrations of the extracts were adjusted to be 1 mg/ml, 5 mg/ml, and 10 mg/ml.

In the antimicrobial activity studies, seven different gram-positive (*Bacillus subtilis* ATCC 6337, *Brevibacillus brevis*, *Bacillus megaterium* DSM 32, *Bacillus subtilis* IM 622, *Bacillus cereus* EMC 19, *Staphylococcus aureus* 6538 P, *Listeria monocytogenes* NCTC 5348), nine different gram-negative (*Salmonella typhimurium* NRRLE 4413, *Pseudomonas fluorescens*, *Enterobacter aerogenes* CCM 2531, *Klebsiella pneumoniae* EMCS, *Escherichia coli* ATCC 25922, *Proteus vulgaris* FMC II, *Pseudomonas aeruginosa* DSM 50070, *Proteus vulgaris*, *Salmonella enterica* ATCC 13311) bacteria were used.

2.7. Anticancer Activity

Human neuroblastoma cancer cells were cultured in an incubator at 37 °C in DMEM F12 nutrient medium containing 12% fetal bovine serum and 0.5% antibiotic (penicillin-streptomycin) in a humidified medium with 5% CO_2. After 24 hours of incubation, anticancer activity results of 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL doses of methanol and ethyl acetate extracts of leaf and petiole calli treated with UV-C rays in 96 well plates were examined. The anti-cancer activities of plant extracts were determined by adding 4 µl of WST-1 viability kit to each well in 96 well plates, waiting for 2 hours, and measuring at 450 nm wavelength in the Elisa Reader device.

2.8. Statistical analysis

The descriptive statistics were given as "mean ± standard deviation" in combinations of MS nutrient medium and added plant growth regulators to ensure callus development for the characteristics discussed in the study. No comparison was made in these combinations. Ten leaf, petiole, and root explants were placed in each culture container, using three replications. The students' test was used to compare cell viability (%) with the control group for each concentration discussed in the study. The statistical significance level was taken as 5%, and the GraphPad package statistics program was used for analysis.

3. RESULTS

3.1. Results on Sterilization and Seed Germination

In this study, it was concluded that the most suitable seed sterilisation for *A. officinalis* seeds is to soak them in commercially available bleach for 30 minutes. After sterilisation, the seeds were cultured on MS nutrient medium which contains 3% sucrose, and solidified with 0.35% gelrite and pH adjusted to 5.8±0.2 using 1N NaOH and HCl. It was determined that the most effective germination was on MS medium + gelrite at a rate of 28%.

3.2. The Results on Callus Growth Rate and Callus Weight

Leaves, petiole, and root parts of *A. officinalis* plants germinated in the sterile environment were used as explant sources. Explants properly separated from the plant were cultured on MS nutrient medium containing different doses of 2,4-D (1, 2 mg/l) and BAP (0.25, 0.50, 0.75 mg/l), 5 combinations each.

The cultures were kept in the climate cabinet for 35 days under suitable conditions. Following a period of 35 days, the callus growth rate and callus weights were determined. The studies were planned as three replications, and the mean and standard deviation values of the data obtained from these replications were calculated and are shown in Tables 1, 2, and 3.

Table 1. The callus growth rate and callus weights in petiole explants.

Plant Growth Regulators		The callus growth rate (%)	The callus weights (mg)
2,4-D (mg/l)	BAP (mg/l)		
1	-	64	324.65±1.43
1	0.25	100	516.24±0.48
1	0.50	51	294.31±0.87
1	0.75	43	254.86±1.73
2	-	78	347.82±0.58
2	0.25	62	320.15±0.47
2	0.50	58	298.37±0.13
2	0.75	47	263.58±1.02

±: refers to the standard deviation values obtained with at least three repetitions.

Table 1 illustrates the callus development rates in petiole explants under different medium compositions. The most effective callus development, reaching 100%, was observed on MS medium supplemented with 1 mg/l 2,4-D and 0.25 mg/l BAP. Following this, the medium containing 2 mg/l 2,4-D exhibited a callus development rate of 78%. The medium with 1 mg/l 2,4-D showed a slightly lower rate of 64%. Similarly, the medium consisting of 2 mg/l 2,4-D combined with 0.25 mg/l BAP displayed a callus development rate of 58%. Further, the medium with 1 mg/l 2,4-D and 0.50 mg/l BAP yielded a rate of 51%. The combination of 2 mg/l 2,4-D and 0.75 mg/l BAP resulted in a callus development rate of 47%. Lastly, the medium containing 1 mg/l 2,4-D and 0.75 mg/l BAP showed the lowest callus development rate at 43%.Although the presence of 2,4-D, was effective on callus development, its use with BAP, a cytokinin, improved callus development. However, the increase in the amount of BAP had a negative effect on callus development. Increasing the amount of 2,4-D from 1 mg/l to 2 mg/l increased the percentage of callus development.

Table 1 presents the weights of calli developed under different nutrient medium compositions. The highest callus weight of 516.24±0.48 mg was observed in the medium containing 1 mg/l 2,4-D and 0.25 mg/l BAP. This was followed by the medium with 2 mg/l 2,4-D, which resulted in a callus weight of 347.82±0.58 mg. The medium containing 1 mg/l 2,4-D yielded a callus weight of 324.65±1.43 mg. Similarly, the medium comprising of 2 mg/l 2,4-D combined with 0.25 mg/l BAP exhibited a callus weight of 320.15±0.47 mg. The combination of 2 mg/l 2,4-D and 0.50 mg/l BAP resulted in a callus weight of 298.37±0.13 mg. Furthermore, the medium containing 1 mg/l 2,4-D and 0.50 mg/l BAP produced a callus weight of 294.31±0.87 mg. The medium with 2 mg/l 2,4-D and 0.75 mg/l BAP showed a callus weight of 263.58±1.02 mg. Lastly, the medium containing 1 mg/l 2,4-D and 0.75 mg/l BAP exhibited the lowest callus weight at 254.86±1.73 mg.

Table 2. Callus growth rate and callus weights in leaf explants

Plant Growth Regulators		The callus growth rate	The callus weights
2,4-D (mg/l)	BAP (mg/l)	(%)	(mg)
1	-	59	257.71±1.39
1	0.25	71	332.54 ±0.78
1	0.50	39	224.62±0.98
1	0.75	29	196.38±1.73
2	-	63	260.48 ±0.46
2	0.25	54	245.93±0.21
2	0.50	41	238.06 ±0.81
2	0.75	34	211.37±0.21

±: refers to the standard deviation value obtained with at least three repetitions.

In leaf explants, the most effective callus development was detected on MS nutrient medium containing 1 mg/l 2,4-D + 0.25 mg/l BAP at a rate of 71%. This is followed by the medium containing 2 mg/l 2,4-D at the rate of 63%, the medium containing 59% of 1 mg/l 2,4-D, the medium containing 2 mg/l 2,4-D + 0.25 mg/l BAP at the rate of 54%, the medium containing 2 mg/l 2,4-D + 0. 50 mg/l BAP at the rate of 41%, the medium containing 1 mg/l 2,4-D + 0. 50 mg/l BAP at the rate of 39%, the medium containing 2 mg/l 2,4-D + 0. 75 mg/l BAP at the rate of 34%, and lastly, the medium containing 1 mg/l 2,4-D + 0. 75 mg/l BAP at the rate of 29% respectively (Table 2).

Although the presence of only one auxin, 2,4-D, was effective on the development of calli from leaf explants, its use with BAP, a cytokinin, was more effective on the rate of callus growth. However, the increase in the amount of BAP used negatively affected the callus growth rate from leaf explants. Increasing the amount of 2,4-D from 1 mg/l to 2 mg/l increased the percentage of callus development in leaf explants (Table 2).

Table 2 presents the weights of calli developed under various nutrient medium compositions. The highest callus weight of 332.54±0.78 mg was observed in the medium containing 1 mg/l 2,4-D and 0.25 mg/l BAP. Following this, the medium with 2 mg/l 2,4-D resulted in a callus weight of 260.48±0.46 mg. The medium containing 1 mg/l 2,4-D yielded a callus weight of 257.71±1.39 mg. Similarly, the medium comprising of 2 mg/l 2,4-D combined with 0.25 mg/l BAP exhibited a callus weight of 245.93±0.21 mg. The combination of 2 mg/l 2,4-D and 0.50 mg/l BAP resulted in a callus weight of 238.06±0.81 mg. Furthermore, the medium containing 1 mg/l 2,4-D and 0.50 mg/l BAP produced a callus weight of 224.62±0.98 mg. The medium with 2 mg/l 2,4-D and 0.75 mg/l BAP showed a callus weight of 211.37±0.21 mg. Lastly, the medium containing 1 mg/l 2,4-D and 0.75 mg/l BAP exhibited the lowest callus weight at 196.38±1.73 mg.

Table 3 presents the results of callus development under different hormone combinations. Out of the various environments tested, callus development was observed in only three hormone combinations, while no callus development was detected in other conditions.

The medium containing 1 mg/l 2,4-D and 0.25 mg/l BAP exhibited the highest callus growth rate of 18%. Following this, the medium with 2 mg/l 2,4-D had a callus growth rate of 8%. Lastly, in the medium containing 1 mg/l 2,4-D, the callus growth rate was observed to be 3%.

Table 3. Callus growth rate and callus weights in root explants.

Plant Growth Regulators		The callus growth rate	The callus weights
2,4-D (mg/l)	BAP (mg/l)	(%)	(mg)
1	-	3	25.74±1.68
1	0.25	18	78.26 ±2.34
1	0.50	-	-
1	0.75	-	-
2	-	8	48.06±0.97
2	0.25	-	-
2	0.50	-	-
2	0.75	-	-

±: refers to the standard deviation value obtained with at least three repetitions.
Root explants were observed to be the most inefficient explants in terms of callus development.

Regarding callus weights, they ranged between 78.26±2.34 mg and 25.74±1.68 mg. The medium with the highest callus weight was determined to be the one containing 1 mg/l 2,4-D and 0.25 mg/l BAP. On the other hand, the lightest calli were developed in the medium containing 1 mg/l 2,4-D (Table 3).

When the callus development rates and callus weights of the petiole, leaf, and root explants were compared with each other, it was observed that the petiole explant was more efficient than the other two explants in terms of callus development rate and callus weight. The best results regarding both callus growth rate and callus weight were obtained from petiole explants. After the development of the calli was observed, UV-C was applied for 15 minutes from a distance of 15 cm in the airflow cabinet while opening the lids of the culture vessels in which the calli developed. After drying the calli growing from the UV-C treated leaf and petiole explants, their extraction was carried out using methanol and ethyl acetate solutions. Extraction from root explants was not performed as there was not enough callus development from root explants.

3.3. Antimicrobial Activity Results

While methanol extracts of leaf and petiole calli were prepared with sterile distilled water, ethyl acetate extractions were prepared using DMSO with final concentrations of 1 mg/ml, 5 mg/ml, and 10 mg/ml. The antimicrobial activities of the solutions of all three concentrations were determined using the disk diffusion method. It was determined that the methanol and ethyl acetate extracts of the calli of the leaf and petiole, solutions of all three concentrations (1, 5, 10 mg/ml) did not have antimicrobial activities against the bacteria used in the study.

3.4. Anticancer Activity Results

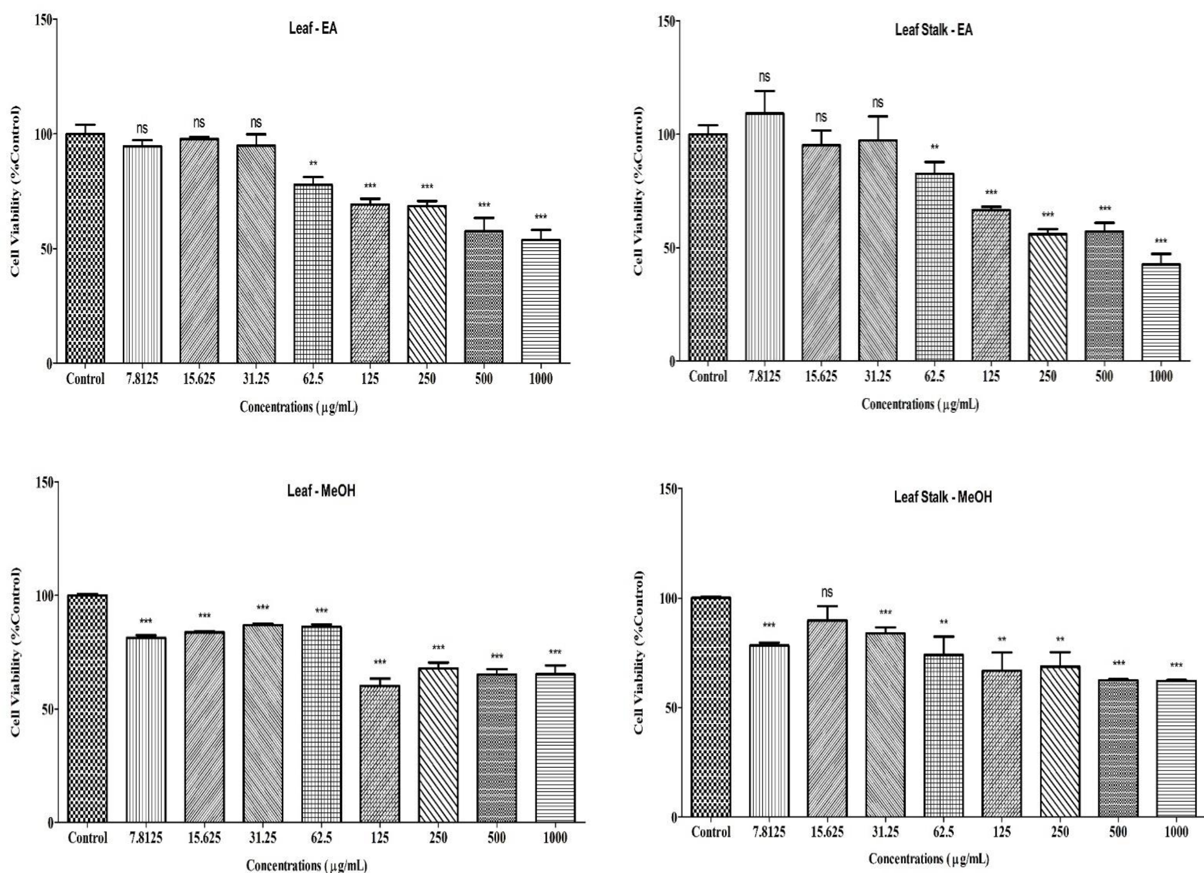
The anticancer activities of methanol and ethyl acetate extracts of calli, of which leaves and petioles were exposed to UV-C treatment, on SH-SY5Y human neuroblastoma cells were determined using the WST-1 viability kit. The anticancer activity results of 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg/mL doses of the extracts are shown in Figure 1.

It was observed that 1000, 500, 250, and 125 µg/mL doses of ethyl acetate extracts obtained from leaf calluses showed a statistically ($p<0.001$) highly significant level of anticancer activity on SH-SY5Y human neuroblastoma cells compared to the control. It was determined that the dose of 62.5 µg/ml had a statistically significant ($p<0.01$) anticancer activity compared to the control. Still, the doses of 31.25, 15.625, and 7.8125 µg/ml did not have any anticancer activity

statistically compared to the control (Figure 1). It was observed that the anticancer activity decreased due to the decrease in the doses of the extracts obtained from the leaf calluses. In other words, as the doses increased, there was an increase in the activity.

It was observed that 1000, 500, 250, and 125 µg/ml doses of ethyl acetate extract of petiole calli showed highly significant anticancer activity on SH-SY5Y human neuroblastoma cells compared to control ($p < 0.001$). It was determined that the 62.5 µg/ml dose had a statistically significant ($p<0.01$) anticancer activity compared to the control, but the 31.25, 15.625, 7.8125 µg/mL doses did not have any anticancer activity statistically (Figure 1). It was concluded that ethyl acetate extracts obtained from the calli grown from leaf and petiole explants showed similar anticancer activities.

Figure 1. Show the effect of the extracts of *Althaea officinalis* L. calli on cell viability in SH-SY5Y human neuroblastoma cells.



The data are represented by the mean ± S.D. from 3 independent experiments and are statistically significant at $p < 0.05$. ^{ns} $p>0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs control treatment, as indicated by the brackets. EA: ethyl acetate; MeOH: methanol.

All doses of methanol extract of leaf calli (1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 µg/mL) showed anticancer activity on SH-SY5Y human neuroblastoma cells. This activity was statistically significant compared to the control group ($p<0.001$). The most effective anticancer activity was obtained using 125 µg/ml of methanol extract (Figure 1).

It was determined that the methanol extract of petiole calli showed anti-cancer activity on SH-SY5Y human neuroblastoma cells at all doses (1000, 500, 250, 125, 62.5, 31.25, 7.8125 µg/mL) except for the dose of 15.625 µg/ml. Compared with the control group, this activity was found statistically significant ($p<0.01$) (Figure 1). It was determined that methanol extracts

of leaf and petiole calluses showed almost similar results in terms of anti-cancer activity. When the anti-cancer activities of methanol and ethyl acetate extracts were compared, it was observed that methanol extracts showed a more effective anti-cancer activity compared to ethyl acetate extracts. The reason for this situation can be explained by the fact that methanol is a more effective organic solvent.

4. DISCUSSION and CONCLUSION

The reason why plants are used in alternative medicine is the valuable secondary metabolites they contain. The different parts of *A. officinalis* contain many components. Pectins, mono and disaccharides, mucilage, flavonoids, isoquercitrin, camphor, caffeic, p-coumaric acid, coumarins, scopoletin, phytosterol, tannins, asparagines are the main components (Al-Snafi, 2013). Previous studies have shown that *A. officinalis* has many pharmacological effects, including antimicrobial, anti-inflammatory, cardiovascular, antiestrogenic, cytotoxic, immunological and immunomodulatory effects (Ahmad *et al*., 1998; Lin *et al*., 1999; Bonjar *et al*., 2004; Guatem *et al*., 2015; Rezai *et al*., 2015; Zhang *et al*., 2016; Twaij *et al*., 2018; Qaralleh *et al*., 2020). Besides, this study, it was determined that methanol and ethyl acetate extracts obtained after UV-C application on calli which were developed using leaves and petiole of *A. officinalis* showed anti-cancer activity.

Our findings are consistent with previous literature on the subject. In a study conducted by Naz and Anis (2012) on the interaction of exogenous hormone concentration and adenine sulfate in *A. officinalis*, three distinct phases of callus formation were investigated. The study presented results obtained from various leaf segments.

As a result of the study, an effective callus development protocol was created by adding 2,4-D at 15 µM concentration in a liquid MS nutrient medium. Our study used 1 and 2 mg/l doses of 2,4-D to ensure callus development. A similar result was obtained using the same plant growth regulator. However, in our study, 2,4-D was used in combination with a cytokinin, BAP, and the amount of use of 2,4-D differs from the aforementioned study. Another similar aspect of both studies is the use of the leaf as an explant source. However, while we used the leaf as an explant source by dividing it in half in our study, different leaf segments were used in the aforementioned study. This makes the two studies different from each other.

In another study on developing an effective shoot regeneration protocol in *A. officinalis* (Naz et al., 2015), the node parts of the plant were used as an explant source. Effective shoot regeneration was obtained by adding BA, Kn, and 2-iP together with combinations of IBA, IAA, and NAA to the MS nutrient medium. In the study, different types of cytokinin and auxin were used to ensure shoot formation and an effective shoot regeneration protocol was obtained. Our study received an effective callus development protocol using BAP, a cytokinin, with 2,4-D, an auxin, at different concentrations. However, the fact that the study is a shoot regeneration and our study is a callus development study separates the two studies from each other. In addition, the explants used in both studies are different from each other.

In another study on the effect of plant growth regulators on *in vitro* production of *A. officinalis* (Mujib et al., 2017), the root, node, and leaf parts of the plant were used as explant source and when 2,4-D was added to the medium, the callus was obtained from explants which were used. The most effective callus growth was obtained from the node explants with a rate of 62%, the leaf explants with a rate of 39%, and the root explants with a rate of 27%. However, not only 2,4-D but also 0.5, 1, and 2 mg/l BAP were added to the medium. Similarly, in our study, an effective callus development protocol was obtained as a result of using 2,4-D and BAP together. Although the plant hormones used in the mentioned study and our study are the same, the concentrations which were used differ from each other. In addition, while the most effective callus growth rate was obtained from the node explant in that study, petiole was

determined as the most efficient explant in terms of callus development in our study. In both studies, root explant was found to be an inefficient explant source in terms of callus development. In the root explants we used in our study, the callus development was observed at 18% as the highest rate while this rate was 27% in the mentioned study.

In a study conducted to determine the antibacterial activities of *A. officinalis* and *A. hirsute* extracts (Lin et al., 1999), it was determined that both plant extracts did not show antibacterial effects. In our study, methanol and ethyl acetate extracts of the calli were made after UV-C application to the calli obtained from the leaf, and petiole. In this sense, the findings of our study and the literature findings support each other.

In another study investigating the antibacterial effects of the methanol extracts of *A. officinalis* and *A. cannabina* (Ahmad et al., 1998), it was observed that both plant extracts produced inhibition. In this study, which was conducted with 52 different bacterial species, very good results were obtained in 17 bacteria. However, the lack of antimicrobial activity of the methanol and ethyl acetate extracts of the calli developed in our study caused the two studies to be different from each other. The disparity in results may be attributed to the contrasting methodologies employed. In the previous study, the plant itself was utilized, whereas in our study, we specifically developed callus from the leaf and petiole of the plant for experimentation. This discrepancy in experimental approaches likely accounts for the observed differences between the two studies. In obtaining such a result, the fact that the biologically active compounds of the plant itself and the calli are different may be effective. For this reason, more detailed studies should be carried out, and the biologically active compounds of the plant and calli collected from the natural growing environment should be compared.

In another study investigating the antimicrobial activities of extracts obtained from *A. officinalis* flowers (Bonjar, 2004), it was emphasized that while an effective antimicrobial activity was observed on some of the used pathogenic microorganisms, there was no antimicrobial activity on others. Likewise, in our study, the determination of the absence of antimicrobial activity of the methanol and ethyl acetate extracts of calli is supported by the literature findings. However, it is important to note that our study differs from the previous one in terms of the methodology employed. While the previous study utilized extracts obtained from the flowers of the plant, our study focused on utilizing callus extracts developed from leaves and petiole, which were then subjected to UV-C treatment. As a result, these differing approaches between the two studies contribute to their variations in findings.In the study in which the antimicrobial activities of ethanol, hexane, ethyl acetate, and water extracts of *A. officinalis* on *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* bacteria were investigated using the methods of disc diffusion and microdilution (Qaralleh et al., 2020). It was determined that the extracts had weak antimicrobial activity. Above all, it was emphasized that the microdilution method is a more effective method for the determination of antimicrobial activity. In our study, the disc diffusion method was correspondingly used in the determination of antimicrobial activity, and a similar result was obtained. The use of a different antimicrobial activity measurement technique such as microdilution in our study might have led to more positive outcomes as a result of the study.

In a study investigating the antimicrobial activities and wound healing potential of hydroalcoholic extracts of *A. officinalis* on clinical strains as well as pathogenic bacteria such as *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and Listeria monocytogenes* in comparison with ciprofloxacin, gentamicin, and penicillin antibiotics (Rezai et al., 2015), disc diffusion and MIC methods were used in antimicrobial activity studies. Consequently, it was emphasized that although the extract was not effective on gram-negative bacteria, it was effective on gram-positive bacteria. The absence of antimicrobial activities of methanol and ethyl acetate extracts obtained from leaf and petiole calluses on both gram-

negative and gram-positive bacteria, which we used in our study, enabled both studies to partially support each other. No antimicrobial activity was detected on gram-negative bacteria in either study. Another difference is that while methanol and ethyl acetate extracts were used in our study, hydroalcoholic extract was used in the current study.

A study that examined the anticancer activity on A549 cells using root extracts of *A. officinalis* (Zhang et al., 2016) stated that the root extract at a dose of 25 mg/ml showed a highly effective anticancer activity. Moreover, in our study, it was determined that both methanol and inert acetate extracts showed very effective anticancer activity, and this suggests that the same biologically active substance or substances may be present in the extracts used. This should be demonstrated using detailed chromatographic techniques. However, we used SH-SY5Y neuroblastoma cancer cells in our study while A549 cells were used in the aforementioned study.

In a study using water extracts of the flower, leaf, and root parts of the *A. officinalis* plant (May & Willuhn, 1985), it was determined that 10% concentrations of the extracts had an inactivation effect on HeLa cells. Our findings also showed that the methanol and ethyl acetate extracts we used had a highly effective inactivation on SH-SY5Y neuroblastoma cancer cells. Both studies differ from each other: they use different cancer cells, use different solvents for extraction, and make use of the different extracted plant parts.

In a study investigating the anti-cancer activities of *A. officinalis* and *A. esculentus* flower and root extracts on HeLa and SK-Hep1 cells (Park et al., 2010), it was observed that both plants have anticancer activities. It was determined that *A. officinalis* showed a stronger anticancer activity compared to *Abelomoschus esculentus*. It was stated that the anti-cancer activity of the extracts obtained by using the whole plant was more effective than the flower and root parts. In our study, it was determined that the callus that was developed by using the leaf and petiole of *A. officinalis* and treated with UV-C showed a very effective anti-cancer activity. In the aforementioned study, the effect of flower and root extracts of *A. officinalis* on HeLa and SK-Hep1 cancer cells was investigated. On the other hand, in our study, the anti-cancer activity of methanol and ethyl acetate extracts of UV-C-treated calli developed using the leaf and petiole of *A. officinalis* on SH-SY5Y neuroblastoma cancer cells was investigated. This situation caused these studies to be different from each other.

In the study in which the methanol extract was obtained by using the aerial parts of *A.ludwigii* L. and its anti-cancer activity against MCF-7 cells was investigated, it was noted that the used plant extract has a highly effective anti-cancer activity against MCF-7 cancer cells. It was pointed out that the plant extract is rich in rutin in terms of its bioactive components, and that rutin, which has a strong anti-cancer activity, may have played a role in the formation of such an effect (Alshaya et al., 2019). It raises such questions: Could this be the reason why the extracts we used in our study have strong anti-cancer activity because they are rich in rutin content? In addition, how does the routine amount change in calli before and after UV-C application? In order to clarify these questions, routine content analyses of calli should be performed before and after UV-C application.

Although the plant in the aforementioned study is in the same genus as the plant we used in our study, the fact that they are different species and the use of different cancer cells in both studies caused these studies to differ from each other.

It was shown that scopoletin (7-hydroxy-6-methoxy coumarin), which is abundant in *A. officinalis*, has anti-cancer activity by destroying the tumoral lymphocyte effect (Ding *et al.*, 2008). Is the reason why the extracts we used in our study showed effective anti-cancer activity because of the high content of scopoletin? This situation can be demonstrated by

comprehensively studying the biologically active ingredient contents of the extracts used in the study.

To conclude, with this study, an effective sterilization protocol was developed to culture the seeds of the *A. officinalis* plant *in vitro*, and then an effective germination environment was tried to be created because germination difficulties were encountered. Leaf, petiole, and root parts of plants grown *in vitro* were used as explant sources and an effective callus regeneration protocol was developed. It was determined that the most effective medium for callus development was MS nutrient medium containing 1 mg/l 2,4-D and 0.25 mg/l BAP, and the most efficient explant for callus development was petiole. Following UV-C application to the obtained calli, methanol, and ethyl acetate extracts were obtained from these calli. The antimicrobial and anti-cancer activities of the extracts were investigated, and it was observed that the used extracts did not have an effective antimicrobial activity but showed strong anticancer activity. Whereas the microorganisms used in the study had a prokaryotic cell structure, the fact that the cancer cells we used in the study had a eukaryotic cell structure may have caused such a result. The fact that we do not know the biologically active component content of the extracts we used in the study creates a deficiency, but it provides a research area for new studies to be made on this subject in the future.

After the routine and scopoletin content analyses were made, the calli used with the normal plant, these two components, even different component contents, were compared with each other before and after the UV-C application. The question of how these components can affect prokaryotic and eukaryotic cells was put forward by this study.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Fethi Ahmet Özdemir: Investigation, Methodology, Resources, Supervision, Visualization, Software, Formal Analysis, and Writing-original draft. **Mesut Turan:** Investigation, Methodology, Resources, Visualization, Software, Formal Analysis

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Antibacterial and antioxidant activity of compounds from *Citrus sinensis* L. peels and *in silico* molecular docking study

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Abstract: The increasing prevalence of drug resistance, adverse side effects of existing antibiotics, and the resurgence of previously known infections have necessitated the search for new, safe, and effective antimicrobial agents. The peels of *Citrus sinensis* L. (300 g) were extracted using maceration and ultrasonic-assisted extraction methods with ethanol, resulting in yields of 20.99 g and 11.5 g (7%, 7.5%), respectively. Silica gel column chromatographic separation of the ethanol extract yielded N-(1,3,4,5-tetrahydroxydecan-2-y1) octanamide (**1**), decanoic acid (**2**), β-sitosterol-3-*O*-β-D-glucopyranoside derivative (**3**), and (z)-ethyl tetradec-7-enoate (**4**). GC-MS analysis of the essential oil detected 7 chemical components accounting for 99.84% of the total composition of which limonene was found to be the predominant constituent (87.5%). *In vitro* antibacterial tests revealed promising zones of inhibition by ethanol extract (12.67±0.58 mm, at 150 mg/mL), compound **4** (15.67±2.88 mm, at 6 mg/mL), and compound **1** (12.00±0.00 mm, at 6 mg/mL) against *E. faecalis*, *S. typhimurium*, and *P. aeruginosa*, respectively, compared to gentamicin (13.00±1.73 mm, 18.00±1.00 mm, and 16.67±1.15 mm, respectively at 10 μg/mL). DPPH radical scavenging activity indicated that compound **1** exhibited an IC_{50} value of 0.05 mg/mL, compared to ascorbic acid's 0.016 mg/mL. *In silico* molecular docking studies revealed that compounds **1** and **3** had the lowest scoring poses against *E. coli* DNA gyrase B enzyme, human peroxiredoxin 5, and *S. aureus* pyruvate kinase, respectively. These findings support traditional applications of *Citrus* peels in treating infectious diseases, particularly against Gram-positive strains, and highlight their potential use as antibacterial ingredients in cosmetics.

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1. INTRODUCTION

Infectious diseases are public health problems and a significant cause of death worldwide. Infections due to pathogenic microorganisms cause severe concern for human health. Increasing cases of drug resistance, unwanted side effects of existing antibiotics, and the

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reappearance of earlier known infections have demanded the need for new, safe, and effective antimicrobial agents (Ayukekbong *et al*., 2017; Riffel *et al*., 2002; Shetty *et al*., 2016). Plants have been utilized to treat a variety of diseases and preserve health in various cultures around the world since ancient times (Akinyemi *et al*., 2016). The World Health Organization (WHO) estimates that around 80% of the population still uses herbal remedies to treat various ailments because of their ease of access, low cost, and lack of adverse effects (WHO, 2004). Traditional medicine is used as a primary source of health care by the majority of Ethiopians because it is culturally rooted, accessible, and economical (Koehn & Carter, 2005).

Citrus sinensis L. Osbeck (Sweet orange) commonly called 'orange' is a member of the Rutaceae family and Aurantioideae subfamily. It is a major source of vitamins, especially vitamin C, a sufficient amount of folacin, calcium, potassium, thiamine, niacin, and magnesium (Angew, 2007). Because of its excellent nutritional content, and source of vitamins, it is now produced practically everywhere in the world as a human food source (Etebu & Nwauzoma, 2014). Orange fruits are the primary source of key phytochemical elements and have long been prized for their healthy nutritional and antioxidant characteristics (Tripoli *et al*., 2007). Numerous compounds with antibacterial, antioxidant, and anti-inflammatory properties were isolated from the peels of various *Citrus* fruits (Friedman *et al*., 2002). The present study aims to identify the chemical components of *C. sinensis* peel extracts and essential oils along with an evaluation of their antibacterial, antioxidant activity, and molecular interaction of the isolated compounds with selected protein targets.

2. Materials and Methods

2.1. Sample Collection and Identification

C. sinensis fruits were collected on February 2, 2022, from the Oromia region, Metehara (Merti) farm, Ethiopia. The plant material was identified and voucher specimen number RY-0001was deposited at the National Herbarium of Ethiopia, Addis Ababa University, Ethiopia.

2.2. Extraction

The extraction of *C. sinensis* peel was done by using cold maceration (by ethanol) and ultrasonic-assisted extraction (UAE) methods. For the maceration method, powdered peels (300 g) were soaked in 2.5 L ethanol (99.9%) for 72 h at room temperature with occasional shaking. The extract was filtered using Whatman filter paper number 1 and concentrated using a Rota evaporator at 40 °C. For the ultrasonic-assisted extraction method, powdered peels (150 g) were soaked in 750 mL ethanol (99.9%), placed in to the ultrasonic bath, sonicated for 30 min at a temperature of 45 °C, and filtered with Whatman filter paper number 1. The extract was concentrated using a Rota evaporator at 40 ^{O}C and kept in sterile vials in the refrigerator until further use.

The essential oil was extracted by hydrodistillation in a modified Clevenger apparatus for 2 hr from powdered *C. sinensis* peels (80 g) in 500 mL of distilled water. The condensate (mixture of essential oil and water) was collected in a 100 mL separatory funnel. The essential oil was consecutively separated from the aqueous layer, dried using anhydrous magnesium sulphate, transferred in to a GC-MS vial, and stored at 4 °C. The yield of essential oil of the *C. sinensis* peel was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{\text{Amount of extracted oil (g)}}{\text{Amount of dry plant material}} \text{ x } 100\%$$

2.3. Isolation of Compounds

The crude extract (15 g) obtained was adsorbed on 15 g of silica gel (mesh size 60-120), subjected to silica gel column chromatography (150 g of silica gel), and eluted with increasing

gradient of ethylacetate in *n*-hexane followed by methanol in dichloromethane. A total of 87 fractions each 100 mL were collected. Fractions that showed similar R_f values and the same characteristic color on Thin Layer Chromatography (monitored by UV lamp at 254 nm and 365 nm) were combined. Fraction 40 (eluted with 100% EtOAc) afforded compound **1** (34.4 mg) with a single spot (R_f value of 0.44) on TLC (eluted with 100% EtOAc). Fraction 32 (eluted with 35% EtOAc in *n*-hexane) afforded compound **2** (11.3 mg). Fractions 41-46 (eluted with 100% EtOAc) were combined and purified by silica gel column chromatography gradient mode of ethyl acetate in *n*-hexane and afforded compound **3** (10.1 mg) (eluted with 30% EtOAc in *n*-hexane). Fraction 19 (eluted with 25% EtOAc in *n*-hexane) yielded compound **4** (34.7 mg).

2.4. Phytochemical Screening of Crude Extract

Phytochemical examinations were carried out on ethanol extract of *C. sinensis* peels using standard methods (Sofowora, 1996; Sofowora, 1993; Williams *et al*., 1973).

2.4.1. *Test for steroid*

Two milliliters of acetic anhydride were added to the *C. sinensis* peel crude extract (0.2 g) which was dissolved in 2 mL of sulphuric acid. A color change from violet to blue to green indicates the presence of steroids.

2.4.2. *Test for terpenoid*

The crude extract (0.2 g) was mixed with 2 mL of chloroform. Then 3 mL of concentrated sulphuric acid was added to form a layer (Salkowski test). A reddish-brown coloration at the interface indicated the presence of terpenoids.

2.4.3. *Test for saponins*

C. sinensis peel crude extract (0.2 g) was dissolved in five milliliters of water and the tubes were shaken vigorously, the formation of a 1 cm layer of foam indicates the presence of saponins.

2.4.4. *Test for flavonoids*

The crude extract (0.2 g) was treated with a few drops of sodium hydroxide solution. The formation of intense yellow color, which becomes colorless with the addition of dilute acid, indicates the presence of flavonoids.

2.4.5. *Test for tannins*

The crude extract (0.2 g) was boiled in 20 mL of water in a test tube. The solution was filtered and a few drops of 0.1% Iron III chloride were added. The appearance of a green/bluish-black color indicates the presence of tannins.

2.4.6. *Test for alkaloids*

One milliliter of 1% HCl was added to 3 mL of the peel crude extract. The mixture was heated for 20 min, cooled, and filtered. Then 1 mL of the filtrate was tested with 0.5 mL Mayer's, reagent (Potassium Mercuric Iodide). The formation of a yellow color precipitate indicates the presence of alkaloids.

2.4.7. *Test for phenol*

Ferric chloride test: the peel crude extract (0.2 g) was treated with 3-4 drops of ferric chloride solution. The formation of bluish-black color indicates the presence of phenols.

2.5. GC-MS Analysis of Essential Oil Extract

GC-MS analysis of essential oil was performed by a GC (7890B, Agilent Technologies, USA) coupled with an MS (5977A Network, Agilent Technologies). The GC had an HP-5MS column (30mm × 250 μm internal diameter (i.d.) and 0.25 μm). Helium was used as a carrier gas (flow

rate 1 mL/ min).The initial oven temperature was 100 °C for 2 min and raised from 100 to 280 ºC at the speed of 10 ºC/min (inlet 250 °C; detector 280 °C; splitless injection/purge time 1.0 min), solvent delay 4.00 min. Mass spectra were recorded in electron-impact mode, with ionization energy of mode at 70 eV, scanning the 33-550 m/z range. The volatile compounds in the oil were identified by comparing the mass spectra of the compounds in the oils with those in the database of NIST11 GC-MS libraries.

The retention indices (RIs) for all of the essential oils were determined by co-injection of the sample with a mixture of the homologous series of C_8-C_{25} *n*-alkanes. Identification of components was based on a comparison of their mass spectra (MS) with those of NIST MS search 2.0 and Wiley 275 libraries and with those described by Adams (Hanuš*et al*., 2008).

2.6. Biological Activity

2.6.1. *Antibacterial activity*

In vitro antibacterial activities of *C. sinensis* peel crude extract and isolated compounds were examined. The antibacterial activities were determined against six pathogenic bacterial strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603) and *Salmonella typhimurium* (ATCC 13311) by agar disk diffusion method. The four sets of dilutions (150, 75, 37.5, and 18.75 mg/mL) of *C. sinenses* peel ethanol extract and isolated compounds (6, 3, 1.5, 0.75 mg/mL) were prepared. The strains of bacteria cells were adjusted at 1.5×10^8 colony forming units (CFU/mL). To determine the number of bacteria present, a 0.5 McFarland standard was prepared by combining 0.05 mL of 1 percent $BaCl_2$ and 9.95 mL of 1 percent H_2SO_4 in distilled water. To contrast a bacterial solution, the preparation was stored in a flask. Mueller-Hinton sterile agar plates were inoculated with indicator bacterial strains.

Sterile filter paper disks (Whatman No. 1, diameter = 6 mm) were placed on the inoculated Mueller-Hinton agar plates. From each dilution, 20 µL were taken and dispensed on the filter papers and allowed to stay at 37 °C for 24 h. Control experiments were carried out under similar conditions, by using gentamicin as positive control and DMSO as a negative control. The zones of growth inhibition around the disks were measured after 18 to 24 h. The sensitivities of the bacterial strains to the peel extracts were determined by measuring the sizes of inhibitory zones starting from the edge of the disk to the edge of the clear zone on the agar surface around the disks, and values < 7 mm were considered as not active against microorganisms.

2.6.2. *Determination of minimum inhibitory concentration (MIC)*

Minimum inhibition concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The broth dilution method was adopted to find minimum inhibitory concentrations (MICs) of active extracts. *C. sinensis* peel crude extract and isolated compounds that exhibited antibacterial activity in the preceding test were chosen. The initial concentration of the crude (5 mg/mL) and isolated compounds (1.00 mg/mL) were diluted using two-fold serial dilution by transferring 1 mL of crude into 1 mL of sterile nutrient broth and mix it into a vial, and then serially diluted it into 5 vials. Each concentration was inoculated with 0.02 mL of the standardized bacterial cell suspension and incubated for 24 h at 37 °C. The turbidity or cloudiness of the broth was an indicator of bacteria growth in the broth (Galma *et al*., 2021).

2.6.3. *Determination of minimum bactericidal concentrations (MBC)*

Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antibacterial that prevents the growth of an organism after subculture onto media. The broth dilution method was used to determine *C. sinensis* peel crude extract, essential oil, and isolated compounds'

minimum bactericidal concentration. A fresh nutrient broth medium was used to transfer samples from the MIC determination set. A loop full of all the tubes used in the MIC, which did not show bacteria growth inoculated and sub-cultured onto sterile nutrient agar media streak plating by a sterile wire loop. Following that, all agar media were incubated at 37 °C for 24 h after the transfer is completed. The test tubes were examined for growth at the end of the incubation period. The MBC of the crude extract against the particular tested strain was recorded at the lowest concentration that exhibited no microbial growth.

2.6.4. *Antioxidant activity*

DPPH solution (0.1 mM) was prepared in methanol by dissolving 0.04 g DPPH in 100 mL methanol. The solution was kept in darkness for 30 min to complete the reaction. Ethanol extract from the peels was diluted in four test tubes 1000, 500, 250, and 125 µg/mL. From this concentration, 1 mL of each was mixed with 4 mL of 0.04% DPPH. For the compounds, the same procedure was performed. The samples were diluted 100, 50, 25, and 12.5 µg/mL. From each concentration, 1 mL of the concentration was mixed with 4 mL of 0.04% DPPH. The resulting solutions were subjected to a UV-Vis spectrophotometer to record absorbance at 517 nm. The percentage of DPPH inhibition was calculated according to the following formula.

$$\text{\% of radical scavenging activity} = \frac{Ab_{standared} - Ab_{analyte}}{Ab_{standard}} \times 100$$

$Ab_{standard}$ is absorbance of standard whereas $Ab_{analyte}$ is absorbance of the analyte.

2.7. *In-silico* Molecular Docking Study of Isolated Compounds

2.7.1. *Preparation of protein and ligand*

3D crystal structures of targeted proteins, *E. coli* Gyrase B (PDB ID: 6F86), *S. aureus* Pyruvate Kinase and Human Peroxiredoxin 5 (PDB ID: 1HD2) were chosen as the protein model and retrieved from the Protein Data Bank (http://www.rcsb.org/). The coordinates of the structures were complexed with water molecules and other atoms which are responsible for increased resolution. Crystal structure of *E. coli* Gyrase B 24 kDa with resolution 1.90 Å has one chain co-crystallized with native ligand 4-(4-bromo-1H-pyrazol-1-yl)-6-[(ethylcarbamoyl) amino]-N-(pyridin-3-yl) pyridine-3-carboxamid ($C_{17} H_{16} Br N_7 O_2$). Crystal structure of *S. aureus* Pyruvate Kinase in complex with a naturally occurring bis-indole alkaloid with resolution: 3.30 Å contains three chains, A, B, C, D, and one native ligand, and four PO_4 groups. (3S, 5R)-3,5-bis(6-bromo-1H-indol-3-yl)piperazin-2-one ($C_{20}H_{16}Br_2N_4O$) found on chain B and D and PO_4 on chain A, B, C, D.

Crystal structure of Human Peroxiredoxin 5, a Novel Type of Mammalian Peroxiredoxin at 1.5 Å Resolution (1HD2) has one chain (A) co-crystalize with benzoic acid ($C_7H_6O_2$) bromide ion (Br^-).Therefore, hetero-atoms (native ligand/ PO_4 groups) to make the active site free for docking and water molecules which may interfere with ligand/receptor binding (Wong & Lightstone, 2011) were removed using discovery studios visualizer and saved in pdb format. Then polar hydrogen atoms and Kollman charges were added for proper optimization (Galma *et al*., 2021) using an MGL tool and saved in PDBQT format.

The structures of test compounds were drawn using chemdraw ultra 8. Energy minimizations of the ligand/compounds were done using chemdraw 3D ultra 8 and stored in .pdb format. Then PDB format of the compounds was treated using MGL, gasteiger charges were added, non-polar hydrogens are merged out and the torsion angle of the ligand was adjusted automatically. Finally, the prepared compound file was saved as PDBQT in the working directory.

2.7.2. *Grid Map preparation and docking*

Compounds **1** and **3** were docked to target proteins *i.e E. coli* DNA gyrase B (PDB ID: 6F86), *S. aureus* Pyruvate Kinase (PDB ID: 3t07), and Human Peroxiredoxin 5 (PDB ID: 1HD2). The active site was identified by studying the interaction between the native ligand and the enzyme. Docking was performed on the active site of the protein. The search was carried out by building a grid box with a volume that is big enough to cover the active site of the protein. Grid box size of 50 ×50× 54 Å points with a grid spacing of 0.375 Å was considered. The docking of proteins was carried out using the Autodock vina program. After the preparation of the configuration file which contains the receptor-name, pdbqt, ligand-name, pdbqt, grid dimension, and exhaustiveness (run repetition) autodock vina run through the command prompt. Docked structure (ligand-protein complex) was prepared using Pymol, and structure analysis was done and visualized using Biovia Discovery Studio 2021.

3. Results and Discussion

3.1. Extract Yield

Peels of *C. sinensis* were extracted using ethanol 99.9% by maceration (300 g) and ultrasonic extraction (150 g) to afford 20.99 g (7%) and 11.5 g (7.5%) yields, respectively, suggesting the ultrasonic extraction (UAE) gave better yield in agreement with previous studies (Saini *et al*., 2019; Safdar *et al*., 2017). In this regard, it can be explained that UAE is based on the principle of acoustic cavitation which is capable of damaging the cell walls of the plant matrix and thereby favoring better yield compared to the maceration technique.

3.2. Phytochemical Screening

Following conventional experimental techniques, phytochemical screening tests were performed to determine the class of secondary metabolites present in the crude extracts. The phytochemical screening tests conducted on ethanol peels extract of *C. sinensis* revealed the presence of alkaloids, flavonoids, tannins, steroids, phenol, and terpenoids whereas saponins were absent (Table 1) suggesting the peels composed of secondary metabolites with significant biological importance.

Table 1. Phytochemical screening results of *C. sinensis* peel crude extract.

Secondary metabolites	Observation
Alkaloid	+
Tannins	+
Flavonoid	+
Steroid	+
Phenol	+
Terpinoid	+
Saponins	-

+ shows presence, -shows absence

3.3. Characterization of Compounds

Silica gel column chromatographic separation of the ethanol extract afforded four compounds **1-4** of which compound **3** was isolated herein for the first time from the species.

Compound **1** (Figure 1) was isolated as a greenish solid with an R_f value of 0.44 (eluted with 100% EtOAc). Its ^{1}H NMR spectrum revealed the existence of amide proton at δ 8.02 (s, 1H), and hydroxyl protons at δ 3.9 integrated for two protons. Four methylene protons were observed at δ 2.1 (H-4 to H-7). Terminal methyl groups were observed at δ 0.9 (H-9' and H-8). Its ^{13}C NMR spectrum displayed a carbonyl group (-CO-) at δ 179.3 attributed to amide carbonyl. Carbon signals resonating at δ 65.3 (C-1''), 33.7 (C-2), 31.8 (C-5'), 29.5, 29.4, 29.2, 28.9, 24.8,

and 22.4 belong to methylene groups, supported by DEPT-135 spectrum pointing down, were visible. The presence of three sp^3 oxygenated methine carbons was observed at δ 73.9 (C-3'), 62.9 (C-2'), and 63.1 (C-4'). The terminal methyl groups appeared at δ 10.1 and 13.5. The above spectral data is in good agreement with the ^{13}C NMR spectral data of N-(1,3,4,5-tetrahydroxydecan-2-y1)octanamide (**1**, Figure 1).

Figure 1. Structures of isolated compounds (**1-4**).

Compound **2** (Figure 1) was obtained as a greenish solid with an R_f value of 0.45 (eluted with 75% EtOAc in *n*-hexane). Its ^{1}H NMR spectral data (Table 2) showed signals that correspond to methylene protons between δ 1.3-1.6 (m, 10H) and signal at δ 2.4 (2H, t). The latter suggests methylene attached to the carbonyl carbon. Terminal methyl protons appeared at δ 0.9 (t, 3H). Its ^{13}C-NMR spectrum showed (Table 2) the carbonyl group (-CO-) of the carboxylic acid at δ 178.7. In addition, peaks at δ 22.7-33.9 correspond to the methylene groups (8 in number) of which the most deshielded one that appeared at δ 33.9 suggests methylene next to carbonyl (C-2). The terminal methyl group appeared at δ 14.1. The above spectral data suggest that the compound is a decanoic acid fatty acid (**2,** Figure 1).

Compound **3** (Figure 1) was isolated as yellowish solid with R_f value of 0.14 (eluted with 30% EtOAc in *n*-hexane). Analysis of the ^{1}H-NMR spectrum ($CDCl_3$, Table 3) demonstrated the presence of six methyl signals at δ 1.0 (s, 1H), 0.9 (d, J = 6.4 Hz, 1H), 0.89 (d, J = 6.5 Hz, 1H), 0.86 (s, 3H), 0.8 (d, J = 1.9 Hz, 1H), and 0.8 (s, 3H). The singlet signal observed at δ 2.4 integrating for two protons suggest the presence of methylene protons adjacent to a carboxyl.

Table 2. ^{1}H NMR and ^{13}C-NMR spectral data of compound **2** ($CDCl_3$, δ in ppm).

Position	Compound **2**		
	^{1}H NMR	^{13}C NMR	Multiplicity
1	-	178.7	Carboxyl
2	2.4 (2H, t),	33.9	CH_2
3	1.6 (2H, m),	24.7	CH_2
4-7	1.3 (8H, m),	29.7, 29.4, 29.2, 29.1	CH_2
8		31.9	CH_2
9		27.7	CH_2
10	0.9 (t, 3H)	14.1	CH_3

Table 3. ^1H NMR and ^{13}C NMR spectral data of compound **3** along with literature reported for β-sitosterol 3-*O*-β-D-glucopyranoside.

Position	NMR data of compound **3**		β-sitosterol 3-O-β-D-glucopyranoside (Peshin & Kar, 2017)		
	^1H-NMR	^{13}C-NMR	^1H NMR	^{13}C NMR	Multiplicity
1	1.0	37.3	1.0 m, 1.40 m	36.8	CH_2
2		29.2	1.58 m, 1.26 m	29.2	CH_2
3	2.4 (d, 2H)	76.1	2.98 m	76.9	CH
4		39.8	1.98 (ddd, 1.98, 12.94, 12.94)	39.3	CH_2
5		140.3		140.4	
6	5.4(t)	122.1	5.35 (t, J = 3.6)	121.2	= CH
7		31.9	1.73 (ddd, J = 2.5, 7.0, 16.0) 1.95 (ddd, J = 16.0, 2.5, 7.0)	31.4	CH_2
8		31.9	1.36 m	31.3	CH
9		50.2	0.85 m	49.6	CH
10		36.7	-	36.2	
11		22.7	1.42 m	20.6	CH_2
12		38.9	1.52 (dd, J = 4.3, 12.37), 1.20 m	38.3	CH_2
13		42.3	-	41.8	
14		56.0	0.95 m	56.1	CH
15		29.7	1.05 m, 1.57 m	28.3	CH_2
16		29.7	1.25 m, 1.85 m	27.8	CH_2
17		55.5	1.20 m	55.4	CH
18	0.70	11.9	0.70 s	11.6	CH_3
19	0.9	19.3	0.94 s	19.1	CH_3
20		34.2	1.40 m	35.5	CH
21		18.8	0.95(d, J = 6.5)	18.6	CH_3
22		37.3	1.20 m	33.3	CH_2
23		29.2	1.25 m	25.4	CH_2
24		45.8	0.94 m	45.1	CH
25		29.2	1.68 m	28.6	CH
26	0.8	19.8	0.87 (d, J = 7.0)	19.7	CH_3
27	0.8	19.0	0.88 (d, J = 7.0)	18.9	CH_3
28		28.2	1.30 m	22.1	CH_2
29	1.0	12.0	0.97 (t, J = 7.5)	11.8	CH_3
1`	3.9	101.2	4.20 (d, J = 7.9)	100.7	CH_2O-
2`		73.6	2.89 (dt, J = 4.5, 8.0)	73.4	CH_3
3’			3.27 (dt, 8.0, J = 4.5, 8.0)	76.9	
4’		70.2	3.00 (dt, J = 4.5, 8.0)	70.0	
5’		76.1	3.06 m(dt, J = 4.5, 8.0)	76.7	
6’	4.0	63.3	4.55 (dd, J = 2.5, 11.77) 4.40 (dd, J = 5.2, 11.77)	62.8	CH_2
1’’		129.3			
2’’ & 6’’		128.2			
3’’ & 5’’		127.8			
4’’		122.0			

The olefinic proton was observed at δ 5.4 (1H, t). Its ^{13}C-NMR (100 MHz, $CDCl_3$, Table 3) spectrum with the aid of DEPT-135 revealed the presence of four quaternary carbon signals observed at δ 174.6, 140.3, 42.3, and 36.7 of which the peaks at δ 174.6 and 140.3 suggest the presence of ester carbonyl and sp^2 quaternary carbons, respectively. Methine signals were observed at δ 56.8, 56.0, 55.5, 50.2, 45.8, 36.1 and 31.9 along with one sp^3 oxygenated methine at δ 70.2 which is a characteristic peak of C-3 methine of steroids. The carbon signals exhibited at δ 39.8, 38.9, 37.3, 34.2, 31.9, 29.7,28.2, 25.00 and 22.7 were due to methylene carbons. The presence of six methyl signals were apparent at δ 19.8, 19.3, 19.0, 18.8, 12.0 and 11.9. The presence of one olefinic methine was observed at δ 122.1. This coupled with the aforementioned sp^2 quaternary carbon at δ 140.3 suggest a characteristics features of sterols with Δ^5 bond. The presence of one glucopyranose moiety is evident from the appearance of one anomeric carbon signal at δ 101.2 along with series of carbon signals at δ 79.6, 76.1, 73.9, 73.6 and 63.3 of which the peak at δ 63.3 suggest oxygenated methylene (C-6') of the glucopyranose moiety. The spectrum revealed a benzoyl moiety peak resonating at δ 174.6, 127.8, 114.5, and 111.0 of which the former suggest ester carbonyl. The above spectral data suggest that the compound is a benzoyl derivative of β-sitosterol-3-*O*-β-D-glucopyranoside (**3**) reported herein for the first time from the species (**3**, Figure 1). This compound was previously reported from flowers of *Viola odorata* L. (Peshin & Kar, 2017).

Compound **4** (Figure 1) was obtained as greenish solid with an R_f value of δ 0.38 (eluted with 25% EtOAc in *n*-hexane). Its ^1H-NMR spectrum (400 MHz, $CDCl_3$, Table 4) showed the presence of olefinic proton signals at δ 5.4 (brs, 2H). The presence of oxygenated methylene signal was observed at δ 4.2 (m, 2H). The signal at δ 1.3 (brs,6H) is a characteristic signal for methylene protons which was supported by the appearance of an intense carbon signal at δ 29.7 in the ^{13}C-NMR spectrum. The triplet signal observed at δ 2.3 is ascribed to methylene protons attached to a carboxyl group. The signal at δ 2.0 (s, 2H) belongs to allylic methylene protons.

Table 4. ^1H NMR and ^{13}CNMR spectral data of compound **4** ($CDCl_3$, δ in ppm).

Position	NMR data of compound **4**		
	^1H-NMR	^{13}C-NMR	Multiplicity
1		179.6	Carboxyl
2	2.3 (t, J = 6.8 Hz, 2H),	34.0	CH_2
3	1.6 (brs, 2H),	24.7	CH_2
4		29.4	CH_2
5		29.7	CH_2
6	2.0 (brs, 2H)	27.2	CH_2
7	5.4 (brs, 1H)	130.0	= CH
8	5.4 (brs, 1H)	129.7	= CH
9	2.0 (brs, 2H)	27.2	CH_2
10		29.4	CH_2
11		29.1	CH_2
12		31.9	CH_2
13		22.7	CH_2
14	0.9 (s, 3H)	14.1	CH_3
1`	4.2 (m, 2H)	65.0	CH_2O-
2`	0.9 (s, 3H)	14.3	CH_3

An up field proton signal at δ 0.9 (s, 6H) is evident for the presence of terminal methyl proton. Its ^{13}C-NMR (100 MHz, $CDCl_3$, Table 4) spectrum with the aid of DEPT-135 revealed a total of 14 carbon signals of which two olefinic methine signals at δ 130.0 and 129.7, and ten methylene signals at δ 34.0, 31.9, 29.7, 29.4, 29.4, 29.3, 29.1, 27.2, 24.7 and 22.7 are clearly evident. Oxygenated methylene was observed at δ 65 (C-1''). The most up field carbon signal

at δ 14.1 accounts for the presence of terminal methyl protons. The existence of ester carbonyl carbon was observed at δ 179.6 (C-1). The above spectral data of the compound is in good agreement with ethyl tetradec-7-enoate (**4**) which was previously isolated from several plants including chloroform extract of stem of *Turraea vogelii* (Hamid *et al*., 2019).

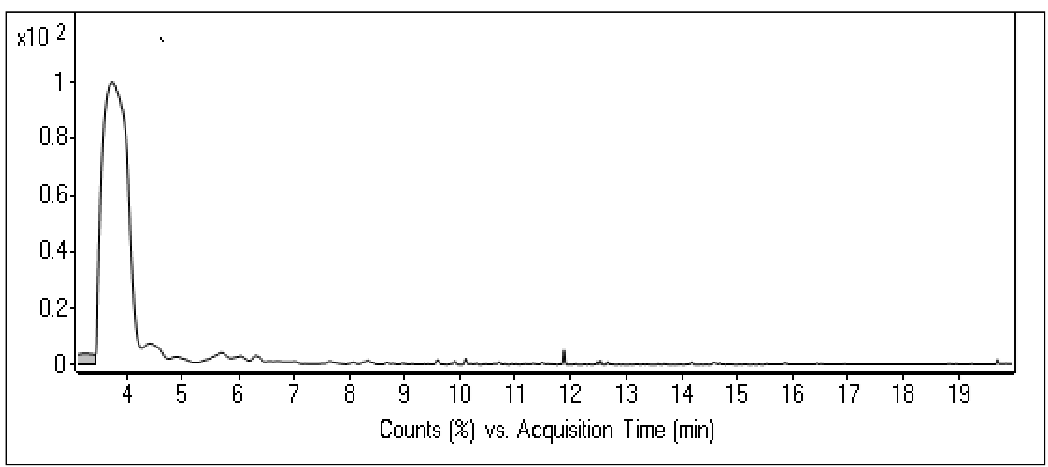
3.4. Essential Oil chemical Composition

The essential oils of *C. sinensis* peel yielded 0.89% (v/w) comparable to literature reports 0.46-2.70% yield from *Citrus* species such as mandarin (2.7%) and orange (0.74%) (Bourgou *et al.*, 2012).The GC-MS analysis of essential oils of *C. sinensis* peel revealed that limonene (87.5 %) was the major constituent followed by 1,6-Octadien-3-ol, 3,7-dimethyl (4.35 %), isopulegol (2.46%), pinene (1.93%), trance-p-Mentha-2,8-dienol (1.45%), carveol P294 (1.25%) and 2-cyclohexen-1-one,2-methyl-5-(1-methylethenyl) (0.98%), sequentially (Table 5, Figure 2). The findings of the present work are in a good agreement with previous reports which reported limonene (70-92.5 %) as a major component of *C. sinensis* peels (Mursiti *et al*., 2019). Uraku *et al.*(2020) reported five compounds from GC-MS analysis of methanolic extract of *C. sinesis* peel such as pyridine-2-carbaldehyde, 1-Methyl-1*H*-pyrrole-2-carbaldehyde (40%), glutamic acid (25%), 2-ethyl-5-methyl-1*H*-pyrrole (14%) and pyrrolidin-2-one (12%).

Table 5. Chemical composition of *C. sinensis* peel essential oils analyzed by GC-MS.

No	Name	RT	Molecular Formula	%
1	Limonene	3.74	$C_{10}H_{16}$	87.5
2	2(10)-Pinene	3.261	$C_{10}H_{16}$	1.93
3	1,6-Octadien-3-ol, 3,7-dimethyl-	4.404	$C_{10}H_{18}O$	4.35
4	trans-p-Mentha-2,8-dienol	4.883	$C_{10}H_{16}O$	1.45
5	Isopulegol	5.704	$C_{10}H_{18}O$	2.46
6	Carveol P294	6.05	$C_{10}H_{16}O$	1.25
7	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-	6.322	$C_{10}H_{14}O$	0.98

Figure 2. GC-MS Chromatogram of *C. sensis* peel essential oils.



3.5. Antibacterial Activity

3.5.1. *In vitro* antibacterial activity

The ethanol extract of *C. sinensis* peels and isolated compounds were tested for their antibacterial activity in different concentrations against six pathogenic bacterial strains, (*E. coli, S. aureus, E. faecalis, P. aeruginosa, K. pneumonia* and *S. typhimurium*) compared to gentamicin as positive control and DMSO as negative control. The mean zone of inhibition

values indicated that all the compounds exhibited a dose-dependent antibacterial activities ranging from 8.00 mm to 15.67 mm. The ethanol extract revealed a promising zone of inhibition against *S. typhimurium* (8.67±1.15 mm), *K. pneumonia* (9.33±0.58 mm) and *E. coli* (9.67±0.58 mm) at 18 mg/mL compared to gentamicin (18.00±1.00 mm, 16.67±2.08 mm and 16.33±1.15 mm, respectively) at concentration of 10 µg/mL. Previous antibacterial activity study of ethanolic extract of *C. sinensis* peel by Nisha *et al*. (2013) revealed 10-16 mm, 11-14 mm, 9-21 mm and 9-18 mm zone of inhibitions against *E.coli*, *K. pneumonia*, *P. aeruginosa*, and *S. typhi* compared to standard antibiotics which is in good agreement with the findings of our study (Table 6).

β-sitosterol 3-*O*-β-D-glucopyranoside derivative (**3**) (6 mg/mL) showed good activity against *K. pneumonia* (11.00±0.00mm) at 0.75 mg/mL compared to gentamicin (16.67±2.08 mm) at 10 µg/mL. Compound **4** (6 mg/mL) showed a good antibacterial activity against *S. typhimurium* (15.33±2.89 mm), and *E. coli* (10.67±0.58 mm) at 0.75 mg/mL compared to gentamicin (18.00±1.00 mm, 16.33±1.15 mm and 16.67±1.15 mm, respectively) at 10 µg/mL. Compound **1** showed a good zone of inhibition against *K. pneumonia* (10.67±0.58 mm) at 0.75 mg/mL compared to gentamicin (16.67±2.08mm) at 10 µg/mL. Compound **2** (6 mg/mL) exhibited promising zone of inhibition against *P. aeruginosa* (11.67±0.58 mm) and *K. pneumonia* (11.00±0.00 mm) at 6 mg/mL compared to gentamicin (16.67±1.15 mm and 16.67±2.08 mm, respectively) at 10 µg/mL (Table 6).

Table 6. Antibacterial activity of *C. sinensis* peel crude extract.

Sample	Conc (mg/mL)	Inhibition Diameter (mm)±SD					
		E. coli	*S. aureus*	*K. pneumonia*	*S. typhimurium*	*E. faecalis*	*P. aeruginosa*
Ethanol extract	150	10.33±0.58	9.00±1.00	11.33±0.58	10.00±0.00	12.67±0.58	8.67±1.15
	75	10.33±0.58	7.67±0.58	10.67±0.58	8.67±1.52	11.00±0.00	8.33±0.58
	37	10.00±1.00	7.33±0.58	9.67±1.15	7.67±0.58	8.33±1.52	8.00±1.00
	18	9.67±0.58	7.00±0.00	9.33±0.58	8.67±1.15	6.33±0.58	7.00±0.00
Compound **1**	6	10.67±0.58	9.67±1.53	10.00±0.00	9.67±1.15	10.00±0.00	12.00±0.00
	3	10.00±0.00	9.00±0.00	9.67±0.58	9.00±0.00	10.00±0.00	11.33±0.58
	45047	10.67±0.58	8.00±1.00	10.33±0.58	9.33±0.58	10.00±0.00	11.00±00
	0.75	10.00±1.00	8.00±1.00	10.67±0.58	9.67±0.58	10.00±0.00	10.00±1.00
Compound **2**	6	9.00±1.00	9.33±1.15	11.00±0.00	10.67±0.58	NA	11.67±0.58
	3	9.00±1.00	9.00±0.00	10.00±0.00	9.67±0.58	NA	9.67±0.58
	45047	8.33±1.53	8.00±1.00	9.00±0.00	8.67±0.58	NA	9.33±0.58
	0.75	8.33±1.15	8.00±1.00	9.00±0.00	8.00±0.00	NA	9.00±0.58
Compound **3**	6	10.33±0.58	NA	10.00±0.00	12.00±1.00	NA	NA
	3	9.00±1.00	NA	10.00±0.00	9.00±1.00	NA	NA
	45047	9.33±0.58	NA	10.00±0.00	9.33±0.58	NA	NA
	0.75	8.33±1.15	NA	11.00±0.00	8.33±1.15	NA	NA
Compound **4**	6	11.33±0.58	9.00±2.00	11.33±0.58	15.33±2.89	NA	11.00±1.00
	3	11.00±1.00	8.33±2.08	11.00±0.00	15.67±1.15	NA	9.00±1.00
	45047	11.00±0.00	8.33±1.52	9.67±1.15	13.00±1.73	NA	8.33±0.58
	0.75	10.67±0.58	8.00±1.15	10.67±0.58	15.67±2.08	NA	8.67±0.58
Gentamycin	10µg/mL	16.33±1.15	18.67±0.58	16.67±2.08	18.00±1.00	13.00±1.73	16.67±1.15

The antibacterial activity of plants is believed to be due to secondary metabolites such as; tannins, terpinoid, phenol, steroid, alkaloid and flavonoids. Tannin observed in *C. sinensis* peel extract has been found to form irreversible complexes with proline rich protein resulting in the inhibition of cell protein synthesis (Shimada, 2006). Flavonoids have antibacterial and antioxidant characteristics (Hodek *et al*., 2002). Terpenoids present in ethanolic extract of *C. sinensis* are thought to play a role in membrane disruption caused by lipophilic substances (Almas *et al*., 2005). The presence of various secondary metabolites in the *C. sinensis* peel could explain for their function as antibacterial agents. The antibacterial activity evaluation of

the present study showed promising antibacterial activity by ethanol extracts of the peels of *C. sinensis* against *K. pneumonia* and *E. faecalis,* compound **1** against *K. pneumonia,* compound **2** against *P. aeruginosa* and *K. pneumoni,* and compound **4** against *S. typhimurium*, *E. coli* and *P. aeruginosa* and this finding agrees with previous studies by Baba *et al*. (2018). Previous studies by Osarumwense (2017) revealed that the methanolic extract of *C. sinensis* peel is highly active against Gram positive, Gram negative bacteria and fungi at concentrations of 100 mg/mL, 150 mg/mL and 200mg/mL, respectively. Similarly, a related study by Omodamiro and Jamoh (2014) suggested that ethanolic extracts at different concentrations (250, 125, 62.5, 31.25, and 15.5 mg/mL) exhibited antibacterial activity against *S. aureus, S. pneumoniae, E. coli, P. mirabilis* and *P. aeruginosa*, in a dose-dependent manner. Thus, the findings of our study are in a good agreement with the previous studies done of the antibacterial activity of various extracts of the peels of *C. sinensis*.

The essential oils showed promising activity against *S. aureus* (10.67±0.58 mm)*, E. coli* (10.67±0.58 mm), *S. typhimurium* (10.67±0.58 mm), *K. pneumonia* (10.33±0.58 mm), *E. faecalis* (10.67±1.15 mm) and *P. aeruginosa* (9.67±1.52 mm) compared to gentamicin (18.67±0.58 mm, 16.33±1.15 mm, 18.00±1.00 mm, 16.67±2.08 mm, 13.00±1.73 and 16.67±1.15, respectively) at concentration of 10 µg/mL (Table 7). Previous studies by Edogbanya *et al*. (2019) revealed higher antibacterial activity of the essential oils against *S. aureus* among the tested bacterial strains which is in a good agreement with the present study. This finding is supported by the results of Burt *et al*. (2004) which showed that Gram-positive bacteria are generally more sensitive to *Citrus* essential oil than Gram-negative. In a related study by Chee *et al*. (2009) and Young *et al*. (2013) limonene is reported to have antibacterial and antifungal properties. Limonene now is known as a significant chemopreventive agent with potential value as a dietary anti-cancer agent in humans (Crowell *et al*., 1994).

Table 7. Antibacterial activity of *C. sinensis* essential oils.

	Conc (mg/mL)	Inhibition Diameter (mm)±SD					
		E. coli	*S. aureus*	*K. pneumonia*	*S. typhimurium*	*E. faecalis*	*P. aeruginosa*
Essential oil	95	12.33±0.58	14.33±1.52	12.00±1.00	12.00±0.00	12.67±0.58	10.67±0.58
	47	12.00±0.00	12.67±0.58	11.67±0.58	11.33±1.15	11.33±0.58	10.67±0.58
	23	11.33±0.58	11.33±0.58	11.33±0.58	11.00±1.00	11.67±0.58	10.33±1.15
	11	10.67±0.58	10.67±0.58	10.33±0.58	10.67±0.58	10.67±1.15	9.67±1.52
Gentamycin 10 µg/mL		16.33±1.15	18.67±0.58	16.67±2.08	18.00±1.00	13.00±1.73	16.67±1.15

3.5.2. *Minimum inhibitory concentration* (MIC)

In the present study, the MIC value obtained from *C. sinensis* peel crude extract, and isolated compounds showed a difference in the inhibitory concentrations against test bacteria (Table 8). The MIC value of the crude extract has been found to be 1.25 mg/mL against *E. coli*, *K. pneumoniae* and *P. aeruginosa* and 2.5 mg/mL against *S. aureus* which is in good agreement with the previous study that revealed MIC value of 1.25 and 2.5 mg/mL against *E. coli* and *P. aeruginosa*, respectively, (Baba *et al*., 2018). Compound **3** showed MIC value (0.25 mg/mL) against *S. typhimurium* and *E. coli,* respectively. Compounds **1** and **2** had the same MIC values at 0.25 mg/mL and 0.5 mg/mL against *P. aeruginosa* and *S. aureus,* respectively. Compound **4** showed MIC value of 1 mg/mL against *P. aeruginosa.*

3.5.3. *Minimum Bactericidal Concentration* (MBC)

MBC values of *C. sinensis* peel crude extract (Table 8) were the same for *P. aeruginosa, E.coli* and *K. pneumoniae* with 2.5 mg/mL value and 5 mg/mL for *S. aureus* which is a better finding compared to that of Baba *et al*. (2018), which reported MBC value of 10 mg/mL, 5 mg/mL

against *S. aureus* and *E.coli*, respectively, and no antibactericidal activity against *P. aeruginosa*. MBC value of the essential oil was similar against *P. aeruginosa* and *S. aureus* with 5 mg/mL value. Compounds **1-3** displayed similar MBC value (0.5 mg/mL) against *P. aeruginosa* and *E. coli.* Compound **1** and **3** showed MBC value (1.00 mg/mL) against *S. typhimurium* and *S. aureus*. Compound **4** showed MBC value (2.00 mg/mL) against *P. aeruginosa* and *S. aureus*.

Table 8. Comparison between MIC and MBC antibacterial values of ethanol extract and compounds.

Bacterial strains	MIC and MBC mg/mL									
	Ethanol extract		Compound **1**		Compound **2**		Compound **3**		Compound **4**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	1.25	2.5					0.25	0.5		
S. aureus	2.5	5	0.5	1	0.5	1			1	2
K. pnumonia	1.25	2.5								
S. typhimurium							0.5	1		
P. arognosa	1.25	2.5	0.25	0.5	0.25	0.5			1	2
E. fecalis	1.25	2.5								

3.6. Antioxidant Activity

Antioxidant activity of the crude extract, and isolated compounds were measured based on their free radical scavenging activity, determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method. By this method, it is possible to determine the radical scavenging power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. As a result of the color changing from purple to yellow, the absorbance was decreased when the DPPH radical is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule (Nishibe *et al*., 1995).Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

Ethanol extract, and isolated compounds of *C. sinensis* peels were tested for their free radical scavenging activities in the DPPH scavenging assay by reacting with stable DPPH radicals. The DPPH radical scavenging activities (%) of ethanol extract, and isolated compounds were found to be 55.69% (compound **1**), 36.15% (compound **2**) 20.7% (compound **3**), 23.8% (compound **4**), 39.94% (crude) at 100 and 1000 µg/mL for the compounds and crude, respectively.

It was observed that the DPPH scavenging activity showed increment in a dose-dependent manner of which compound **1** exhibited the highest percent inhibition of the DPPH compared to ethanol extract and isolated compounds. Ascorbic acid, positive control (µg/mL), showed maximum scavenging effect at very low and high concentration 78, 83, 91% at 25, 50 and 100 µg/mL. Antioxidants have the capability to slow down or prevent the oxidation process of other molecules. In general when compared to standard ascorbic acid, the DPPH radical scavenging activity of ethanol extract of *C. sinensis* peels was found to be lower (Table 9), nevertheless, the activity displayed by the extract increased in a dose dependent which is in a good agreement with previous studies (Akinyemi *et al*., 2016). Based on data in Table 9, the lowest IC_{50} value was displayed by ascorbic acid (0.016 mg/mL), followed by compound **1** (0.5 mg/mL), compound **2** (0.212 mg/mL), compound **3** (50 mg/mL) and compound **4** (2000 mg/mL).

Table 9. DPPH radical scavenging activities (%) of the isolated compounds.

Conc (µg/mL)	Control	Compounds									
		Compound **4**		Compound **3**		Compound **2**		Compound **1**		Ascorbic Acid	
		A	%RSA	A	%RSA	A	%RSA	A	%RSA	A	%RSA
100	1.029	0.784	23.81	0.657	36.15	0.816	20.7	0.702	55.69	0.085	91.74
50	1.029	0.804	21.87	0.736	28.474	0.834	18.95	0.631	49.95	0.168	83.67
25	1.029	0.831	19.24	0.744	27.7	0.839	18.46	0.572	31.2	0.22	78.62
12.5	1.029	0.873	15.16	0.778	24.39	0.883	14.19	0.667	25.56	0.67	34.89
IC_{50} (mg/mL)		50		0.212		2000		0.05		0.016	

Conc (µg/mL)	Control	Ethanol extract		Ascorbic acid	
		A	%RSA		
1000	1.029	0.618	39.94	0.04	96.11
500	1.029	0.71	31	0.045	95.63
250	1.029	0.745	27.6	0.047	95.43
125	1.029	0.825	19.83	0.05	95.14
IC_{50} (mg/mL)		3251.785			

Conc-Concentration, A- Absorbance, %RSA- Radical Scavenging Activity (%)

3.7. *In silico* Molecular Docking

Two compounds were docked in this study against three organism targets proteins in order to predict their orientation and binding affinity at the active site of the receptor. For each compound nine poses were generated with different binding energy and RMSD value. But the dock pose with least binding energy and RMSD value has the highest affinity are considered as the best docked conformation (Azam & Abbasi, 2013).

3.7.1. *Docking of against E. coli DNA gyrasee B enzyme*

Compounds **1** and **3** were docked against *E. coli* DNA gyrase B (PDB ID: 6F86) enzyme and displayed binding affinity of -6.2 Kcal/mol and -6.9 Kcal/mol, respectively, compared to gentamicin (-7.3 Kcal/mol) (Figure 3a, b, Table 10).

Table 10. Compound **1**, **3** and gentamicin binding energy with *E. coli* DNA gyrase B(PDB ID: 6F86).

Ligands	Affinity (kcal/mol)	H-bond	Residual amino acid interactions	
			Hydrophobic/Pi-Cation	Van der Waals
1	-6.2	GLU-50	ALA-47, A: VAL-43, A: ILE-78and A: ILE-94	VAL-71, A: VAL-167, A: THR-165, A: ASP-73, A: ASN-46, A: PRO-79, A: ASP-49, and A: ARG-76, GLY-76
3	-6.9	ASP-73, GLY-77	ARG-76 and A: ALA-53, and A: ILE-94	VAL-93, VAL-120, VAL-97, LEU-98, SER-121, VAL-188, GLY-119, GLY-117, PRO-79 and ASN-46, ALA-47, THR-165, GLU-50, ASP-49, ILE-78
Gentamicin	-7.3	GLY-77, GLU-30, THR-165, ASP-73, ARG-46	PRO-79, ILE-94	ASP-49, ALA-47, ILE-78, GLY-164, GLY-73, ARG-76

Figure 3a. Interaction of compound **1** on *E. coli* DNA gyrase B (PDB ID: 6F86) ((A) 2D interaction, (B) 3D interaction).

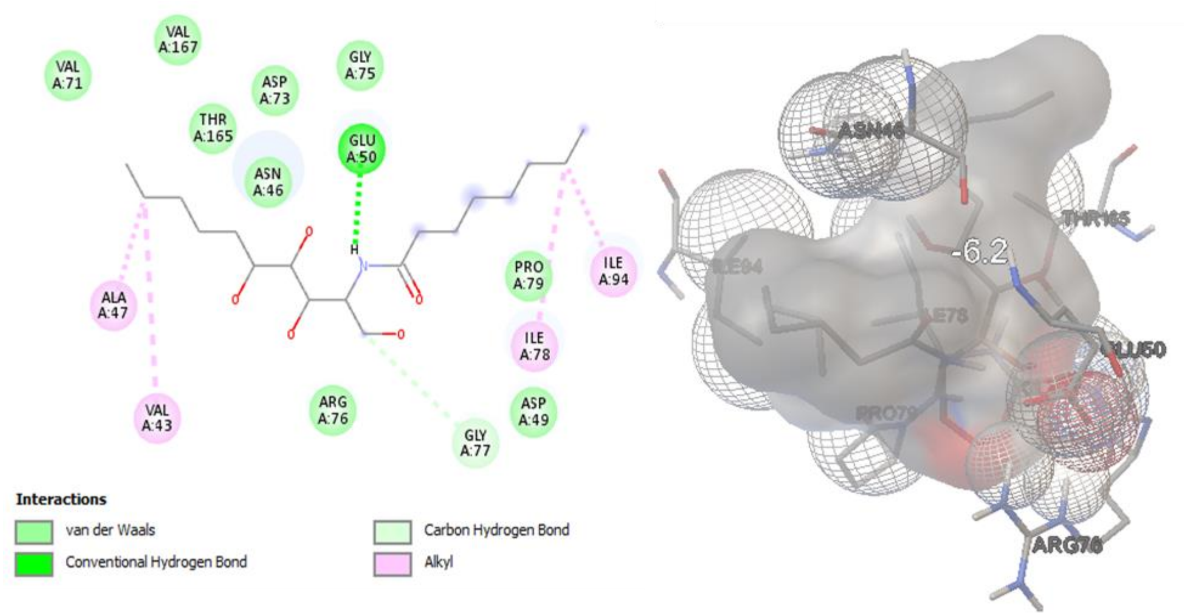
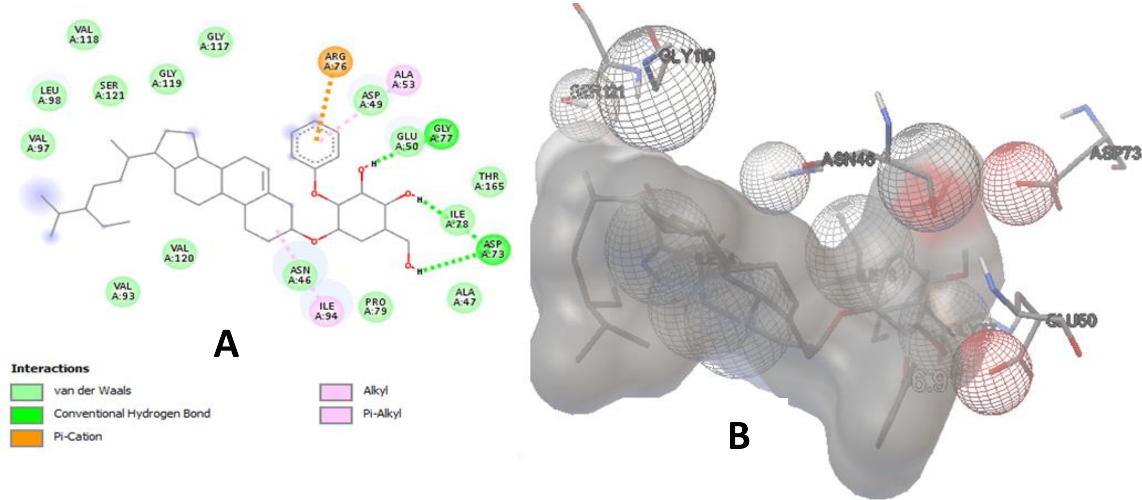


Figure 3b. Interaction of compound **3** on *E. coli* DNA gyrase B (PDB ID: 6F86) (A) 2D interactions (B) 3D interactions.



3.7.2. *Docking against human peroxiredoxin* 5

Compounds **1** and **3** were docked against Human Peroxiredoxin 5(PDB ID: 1HD2) enzyme and displayed binding affinity of -5 Kcal/mol and -7.1 Kcal/mol, respectively, compared to ascorbic acid (-5.6 Kcal/mol) (Figure 4a, b; Table 11).

Figure 4a. Interaction of compound **1** against Human Peroxiredoxin 5 (PDB ID: 1HD2) (A) 2D interactions (B) 3D interactions.

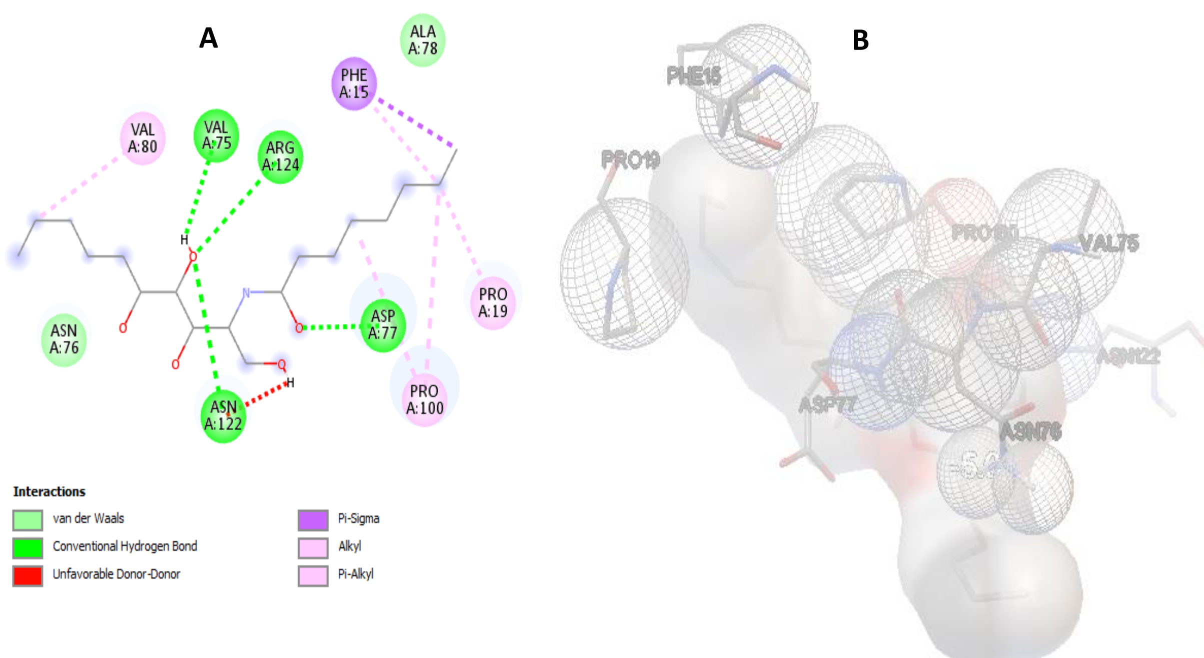


Figure 4b. Interaction of compound **3** against Human Peroxiredoxin 5 (PDB ID: 1HD2) (A) 2D interactions (B) 3D interactions.

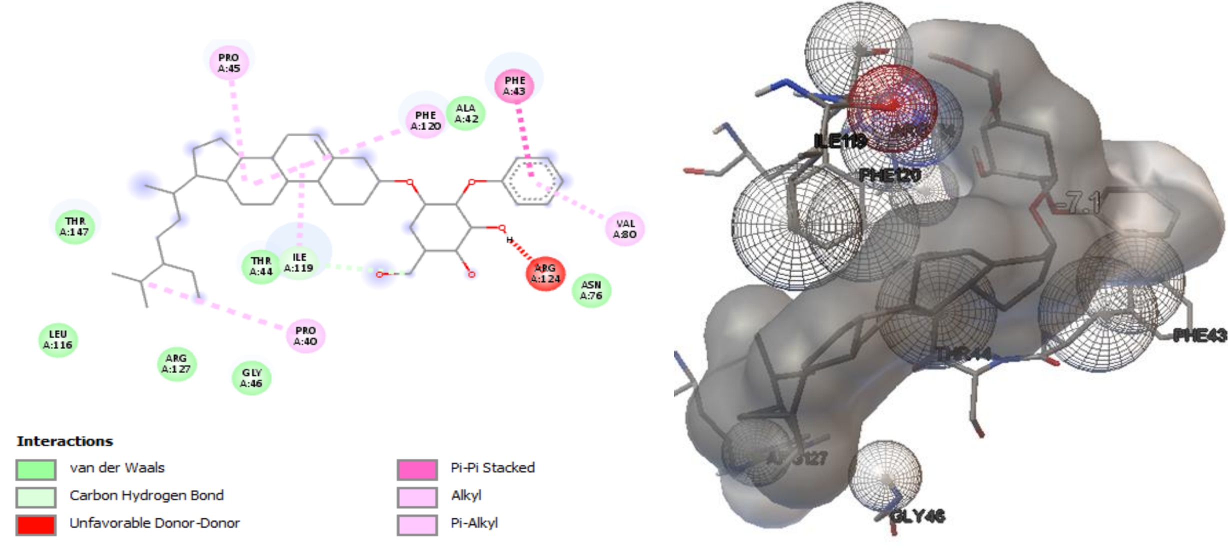


Table 11. Different binding energy and RMSD value of compound **1**, **3** and ascorbic acid against Human Peroxiredoxin 5 (PDB ID: 1HD2).

Ligands	Affinity (kcal/mol)	H-bond	Residual amino acid interactions	
			Hydrophobic/Pi-Cation	Van der Waals
1	-5	VAL-75, ARG-124, ASN-122, ASP-77	PHE-15, VAL-80, PRO-19, PRO-100	ASN-76, ASN-78, GLU-50
3	-7.1		ARG-124, VAL-80, PRO-40, PRO-45, PHE-120, PHE-43	THR-147, LEU-116, ARG-127, GLY-46, THR-44, ASN-76, ALA-42
Ascorbic acid	-5.6	ARG-86 GLY-82 GLU-91	-	GLU-16, LYS-93, GLY-92, VAL-94, ARG-95, ALA-90, GLY-17, GLY-85

3.7.3. *Docking against of S. Aureus pyruvate kinase*

Compounds **1** and **3** were docked against *S. aureus* Pyruvate Kinase (PDB ID: 3t07) enzyme and displayed binding affinity of -6.6 Kcal/mol and -8.2kcal/mol, respectively, compared to gentamicin (-7.7 Kcal/mol) (Figure 5a, b; Table 12).

Table 12. Different binding energy and RMSD value of compound **1**, **3** and gentamicin against *S. aureus* Pyruvate Kinase (PDB ID: 3t07) protein target.

Ligands	Affinity (kcal/mol)	H-bond	Residual amino acid interactions	
			Hydrophobic/Pi-Cation	Van der Waals
1	-6.6	GLN-574, LEU-471, ILE-469, THR-463, HIS-470, ASN-465, LYS-468, GLU460	PHE-578, LEU -471	GLY-462, THR-461, PRO-457, VAL-456, LEU-355, ASP-571, ALA-582, ASN-583, LEU-449, GLU-475, LYS-576, ASP-474, GLY-473
3	-8.2	LEU-269	LYS-390, ALA-573, Val-356	THR-461, GLY-462, THR-463, SER-383, ASP-237, LYS-271, ARG-386, ASN-357, GLY-270, ARG-484, GLY-575, GLY 481, GLY 479, GLN-480, LYS-576, GLN-574, ALA-572
Gentamicin	-7.7	LYS-271, ASP-237, GLU-234, ARG-386, THR-387	ALA-374, VAL-336	GLN-374, VAL-233, GLY-483, ILE-482, LEU-343, ASN-337, SER-383, THR-463, SER-236

Figure 5a. Interaction of compound **1** against *S. aureus* Pyruvate Kinase (PDB ID: 3t07) protein target(A) 2D interactions (B) 3D interactions.

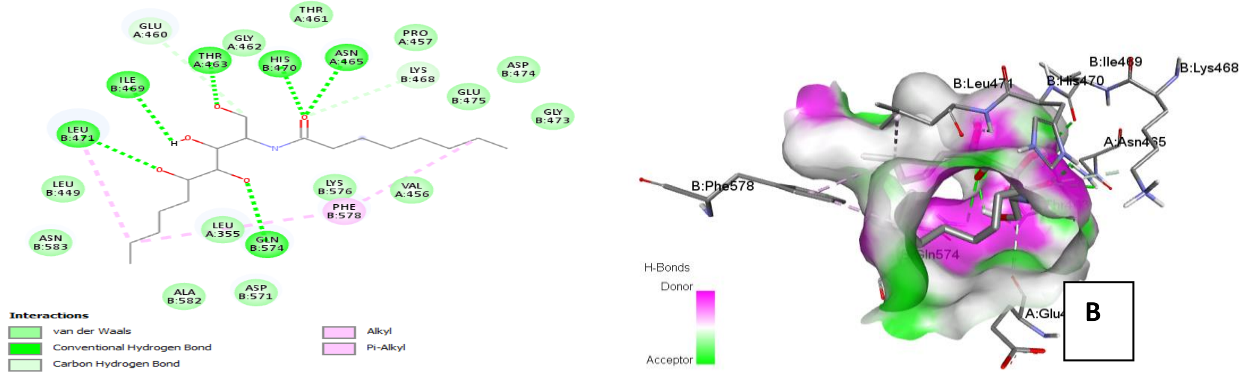
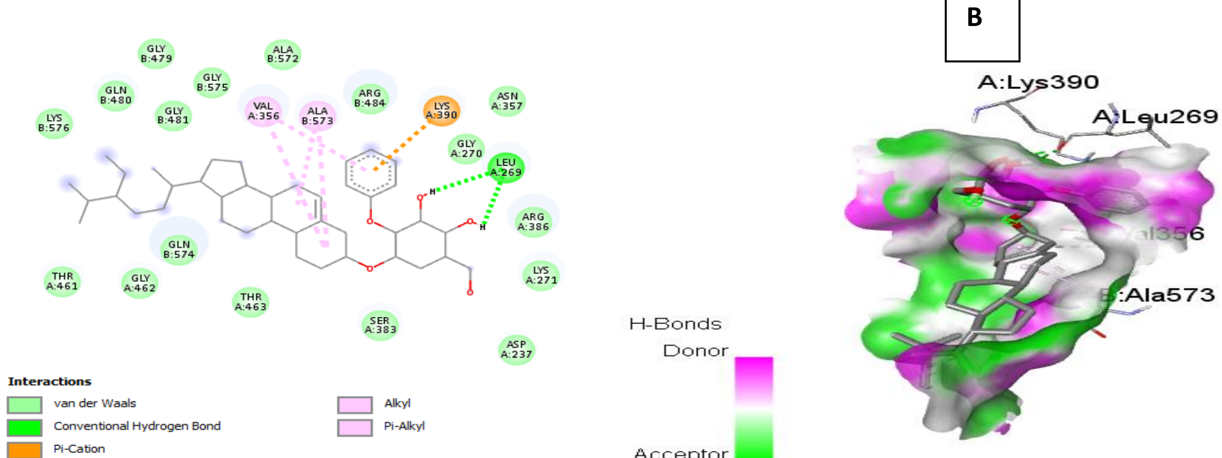


Figure 5b. Interaction of compound **3** against *S. aureus* Pyruvate Kinase (PDB ID: 3t07) protein target(A) 2D interactions (B) 3D interactions.



5. CONCLUSION

The presented study identified *N*-(1,3,4,5-tetrahydroxydecan-2-y1) octanamide (**1**), decanoic acid (**2**), β-sitosterol 3-*O*-β-D-glucopyranosidederivative (**3**) and (z)-ethyl tetradec-7-enoate (**4**) compounds from ethanol peels extracts of *C. sinensis* of which compound **3** was identified herein for the first time from the species. The essential oils from peels of *C. sinensis* peel were analyzed by GC-MS which revealed the presence of seven chemical components accounting for 99.84 % of the total compositions of which limonene (87.5%) was the major constituent followed by 1,6-Octadien-3-ol,3,7-dimethyl- (4.3%), isopulegol (2.46%), pinene (1.9%), and trance-p-Mentha-2,8-dienol (1.45%), respectively. The therapeutic effects of limonene have been extensively studied, proving antioxidant, anticancer, antifungal, antiviral, and gastroprotective effects, among other beneficial effects in health. Thus, the high composition of limonene in essential oils from peels of *C. sinensis* suggest the potential of the oils as food additives and cosmetic ingredients.

The ethanol extract revealed a promising zone of inhibition against *S. typhimurium* (8.67±1.15 mm), *K. pneumonia* (9.33±0.58 mm) and *E. coli* (9.67±0.58 mm) at 18 mg/mL compared to gentamicin (13.00±1.73 mm, 18.00±1.00 mm, 16.67±2.08 mm and 16.33±1.15 mm, respectively) at concentration of 10 µg/mL in agreement with previous study by Najimu (2013). Compound **1** also displayed promising antibacterial activity against K. *pneumonia,* compound **2** against *P. aeruginosa* and *K. pneumoni* and compound **4** against *S. typhimurium* and *E. coli*. The high antibacterial activity of the ethanol extract may be attributed to the synergistic effects of these constituents present in the extract. The essential oil of *C. sinensis* peels had promising antibacterial activity against *S. aureus* (10.67±0.58 mm) and *E. faecalis* (10.67±1.15 mm) at 11 mg/mL compared to gentamicin (18.67±0.58 mm and 13.00±1.73 mm, respectively) at concentration of 10 µg/mL. DPPH radical scavenging activity revealed that compound **1** showed IC_{50} value of 0.05 mg/mL compared to ascorbic acid 0.016 mg/mL indicating the potential of the plant as natural antioxidant remedies.

Compounds **1** and **3** were docked against *E. coli* DNA gyrasee B, Human Peroxiredoxin 5 and *S. aureus* Pyruvate Kinase enzymes and displayed binding affinity of -6.2/-6.9 Kcal/mol, -5/-7.1 Kcal/mol, and -6.6/-8.2 Kcal/mol, respectively, compared to gentamicin (-7.3 Kcal/mol), and ascorbic acid (-5.6 Kcal/mol), respectively. Therefore, the *in vitro* antibacterial, DPPH radical scavenging activity along with the molecular docking analysis suggest the potential use of the peels of *C. sinensis* as promising antibacterial agents which corroborates the traditional uses of the peels of the plant. Moreover, the findings of this study suggest the potential of essential oils as antibacterial ingredients in cosmetic applications.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Raey Yohannes: Conducted experimental work and wrote the original draft. **Milkyas Endale (PhD)** and **Teshome Geremew (PhD):** Supervised the experimental work and edited the manuscript. **Tarekegn Tafese:** conducted computationally analysis.

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