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Table of Contents

Research Articles

Total phenolic content and antioxidant activity of extracts obtained from tobacco waste seeds, grown under organic production
Page: 408-420 **PDF**
Liliya Stoyanova, Maria Angelova Romova, Margarita Docheva, Desislava Kirkova

Biological control of pathogenic fungi using Pseudomonas brassicacearum isolated from Aronia × prunifolia (Marshall) Rehder roots
Page: 421-434 **PDF**
Luau Burhan Mustafa, Ahmed Ismael Naqee Al-bayatı, Dunya Albayati, İbrahim Özkoç

Assessment of the differences of hematological variables and their correlation with glycemic control among type 2 diabetes mellitus patients in Iraq: Comparative cross-sectional study
Page: 435-444 **PDF**
Osamah Ahmed

Carotenoid content, phytochemical screening, and antioxidant potential of Kantutay (Lantana camara L.), Katuray (Sesbania grandiflora L.), and Blue Ternate (Clitoria ternatea L.) flowers in the Philippines
Page: 445-461 **PDF**
Melanie D. Piedad, Lexter Natividad

Selenium nanoparticles synthesized via green methods from Calluna vulgaris extract: Exploring their antioxidant and antibacterial activities
Page: 462-471 **PDF**
Ecem Erdem, Çiğdem Aydın Acar

Secondary metabolites of Santolina africana: chemical profiles and assessment of biological activities
Page: 472-485 **PDF**
Marwa Khammassi, Sana Khedhri, Awatef Slama, Meriam Boudkhılı, Ismail Amrı, Lamia Hamrounı, Bassem Jammoussı

The preliminary study on the antifungal effect of Kaffir lime (Citrus hystrix DC) peel extract against Malassezia furfur
Page: 486-493 **PDF**
Mulyati Sri Rahayu, Wizar Putri Mellaratna, Nailah Najah

Antibacterial and antioxidant properties and phytochemical screening of Laurus nobilis L. extract from Ethiopia
Page: 494-506 **PDF**
Rebecca Beyene, Teshome Biru, Prof. Aman Dekebo

Chemical content profile and antioxidant activity of Rhododendron ponticum L. (Ericaceae) extracts
Page: 507-513 **PDF**
Yusuf Sıcak, İrfan Öztürk, Bihter Şahin, Dilaycan Çam, Cansel Cakır

Effective inhibition of bacterial sialidases by phenolic acids and flavonoids

Page: 514-521 **PDF**

Yana Gocheva, Milena Nikolova, Stephan Engıbarov, Irina Lazarkevich, Rumyana Eneva

Chemical composition and potent antibacterial activities of colony-forming cyanobacteria, Desmonostoc muscorum (Nostocales, Cyanophyceae)

Page: 522-532 **PDF**

Eldrin Arguelles

Effects of nitric oxide on composition of the isolated essential oil from Satureja hortensis L. (Lamiaceae), under the cadmium stress

Page: 533-542 **PDF**

Iraj Azizi, Mohammad Asadi

Antimicrobial and antibiofilm studies on three endemic species of Verbascum L. (Scrophulariaceae) in Türkiye

Page: 543-550 **PDF**

Pervin Soyer, Sevim Küçük, Yağmur Tunalı

Cytotoxic effect of Eleutherococcus trifoliatus (L.) S.Y. Hu stem bark extracts on Gastric, Lung, and Hepatocellular cancer cells

Page: 551-561 **PDF**

Nguyen Quan, Bui Thi Kim Ly, Hoang Thanh Chi

Chemical characterization and antioxidant activities of essential oil of Ficus elastica Roxb. ex Hornem. leaves

Page: 562-573 **PDF**

Chika Attama, Lawrence Luka, Chidama, Bulama Ndakudu

Molluscicidal activity of Nigella sativa seed, Azadirachta indica leaf and Khaya senegalensis bark ethanolic extracts against Bulinus truncatus snails

Page: 574-583 **PDF**

Ayat Alrasheid, Gokhan Zengin, Saad Mohammed Hussein Ayoub, Ahmed Saeed Kabbashi

Examination of the chemical composition and anti-diabetic activities of the oils of Abelmoschus esculentus, Peganum harmala, and Aquilaria agallocha cultivated in Muğla

Page: 584-591 **PDF**

Yunus Çetintaş, Ayşe Çetintaş, Yusuf Sıcak, Mehmet Öztürk

Phytochemical screening and in vitro biological activity of Amaranthus viridis growing in Northern Cyprus

Page: 592-603 **PDF**

Melis Sümengen Özdenefe, Fikret Büyükkaya Kayış, Ümit Haydar Erol, Aysun Mercimek Takcı

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Research Article

Total phenolic content and antioxidant activity of extracts obtained from tobacco waste seeds, grown under organic production

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Abstract: This study aimed to determine the total phenolic content of Oriental tobacco waste seeds, grown under organic production, and to evaluate their antioxidant activity by extraction with different solvents under different conditions. The extraction was performed with H_2O, 99.9% CH_3OH, 60% CH_3OH, and 96% C_2H_5OH under maceration and ultrasonic extraction at 20°C and 40°C. All solvents were used in a volume of 4 mL, 5 mL, and 6 mL. The total phenolic content varied between 0.89 mg/g GAE (maceration; sample/solvent ratio 0.1g/5mL, C_2H_5OH) and 5.85 mg/g GAE (maceration; sample/solvent ratio 0.1g/6mL, C_2H_5OH). Ethanolic and 60% methanolic extracts had the highest antioxidant activity as determined by the DPPH method; 60% methanolic and water extracts had the highest antioxidant activity as determined by the ABTS method; while methanolic and 60% methanolic extracts had the highest antioxidant activity as determined by the FRAP method. In addition, the content of nicotine in tobacco seed extract was not detected.

1. INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a plant from the genus *Nicotiana*, family Solanaceae, and its leaves are widely used for smoking, chewing, and sniffing. *Nicotiana tabacum* is cultivated as an economically important crop all over the world. In Bulgaria, tobacco is produced conventionally according to standard agro-technological practices for the respective variety. In 2016, the Tobacco and Tobacco Products Institute at the Agricultural Academy in Sofia certified a biofield at the Experimental Tobacco Station Department - Gotse Delchev, where tobacco cultivation began under organic farming conditions. Organic production (farming) includes cultivation practices without the use of conventional techniques and plant protection preparations. This is a new worldwide direction that aims at achieving sustainable and ecological agricultural practices (Bozukov *et al.*, 2019; Raei & Aghaei-Gharachorlou, 2015).

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Tobacco plants produce extremely large quantities of seeds that can be collected per hectare area and may change depending on the place, the type of tobacco plant, and weather conditions. The quantity obtained, for example in Türkiye and Macedonia, is from 600 up to 2500 kg per hectare (Usta, 2005). Tobacco seed is also part of the tobacco cultivation process. Seeds are not collected as a commercial product; they are collected for cultivation (Usta *et al*., 2011). Tobacco seeds can be considered waste under certain circumstances, typically when they are no longer needed for cultivation and are discarded. Research into the quality of the tobacco seed by Tomov *et al.* (1983) shows a certain influence of the size of tobacco seeds on their sowing qualities. Seeds smaller than 0.5 mm were of reduced quality and were recommended as unfit for sowing. Kochev (2008) examined the aerodynamic characteristics of tobacco seeds, techniques, and principles of separation for granulation purposes and identified tobacco seeds smaller than 0.5 mm in size as waste.

Tobacco seeds can give two main products, namely cake and oil. The former is rich in proteins (18 – 41%), amino acids (aspartic acid about 2%, glutamic acid 5 – 6%, arginine over 3% as well as serine, isoleucine, threonine, valine, and others not exceeding 2%), and fiber 11 – 12% (Abbas Ali *et al*., 2008; Frega *et al*., 1991; Rossi *et al*., 2013). On the other hand, the oil content of the seed is between 30% – 50%, rich in unsaturated fatty acids, sterols, and tocopherols (Popova *et al*., 2018; Zlatanov *et al*., 2007). Polyphenolic compounds such as gallic acid, 3,4-dihydroxybenzoic acid, and catechin are also found in tobacco seeds (Özcan *et al*., 2023). Data on the content of rutin 0.22 – 0.64 mg/g and chlorogenic acid 0.98 – 2.66 mg/g was found in the literature for Virginia and Burley tobacco seeds. These compounds as secondary metabolites in tobacco seeds have been the subject of study regarding their synthesis as a result of abiotic factors. The phenolic acids of plant extracts can be determined as a total phenolic content (TPC). Plant phenolic compounds are normally soluble in polar organic solvents such as water, ether, chloroform, ethyl acetate, methanol, and ethanol. Methanol, ethanol, and water are mostly used for dissolving phenolics for analytical purposes from different parts of the plant (Alara *et al.,* 2021). The extraction of phenols can be carried out by ultrasound under supercritical CO_2 extraction or microwave-assisted extraction (Banozic *et al.,* 2020). The total phenolic content of tobacco seeds was reported to be 1.24 – 2.26 mg GAE/g when the polyphenols were extracted with CH_3COCH_3 and 1.20 – 1.44 mg GAE/g using 80% C_2H_5OH (Xie *et al*., 2011). Polyphenols are chemical substances with proven antioxidant properties (Kumar *et al*., 2023).

There is little information about the antioxidant activity of extracts from tobacco seeds, especially for those grown under organic production and considered as waste products. In this regard, the scientific interest of the group is directed to determine the total phenolic content in tobacco seeds, considered as waste and grown under organic production. The scope is also to evaluate their antioxidant activity by extracting them with different solvents under different conditions. The aim of this study is therefore to investigate the total phenolic content and antioxidant activity of extracts obtained from tobacco waste seeds, grown under organic production. The data from this study will add to the information on the influence of organic production on the accumulation of phenolic compounds in tobacco seeds. Tobacco seeds do not contain nicotine, a toxic alkaloid with a high content in the tobacco leaves. The results obtained for the antioxidant activity of the compounds extracted from tobacco seeds can outline prospects for their use in the pharmaceutical and food industry as nicotine-free products with health benefits.

2. MATERIAL and METHODS

2.1. Samples

Tobacco seeds from the Bulgarian oriental tobacco variety Krumovgrad 58 were used. The tobacco was grown under organic production conditions in a certified experimental field Gotse Delchev at the Tobacco and Tobacco Products Institute, Plovdiv, Bulgaria under "Technology for organic tobacco production" developed at the Institute of Tobacco and Tobacco Products

(Bozukov, 2018). The seeds were collected and mechanically fractionated with sieves with a diameter of 0.6 mm. Three fractions were separated according to the techniques and principles for separation for granulation purposes described by Kochev (2008) – over 0.6 mm, between 0.5 – 0.6 mm, and under 0.5 mm. Seeds with a size under 0.5 mm were unsuitable for transplanting and considered as waste. The tobacco waste seeds (TWS – under 0.5 mm) were grounded and used for the preparation of extracts.

2.2. Reagents

2,2-diphenyl-1-picrylhydrazyl - DPPH (CAS No: 1898-66-4), 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) - ABTS (CAS No: 30931-67-0), methanol (CAS No: 67-56-1), Gallic acid (CAS No: 149-91-7), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ethanol (CAS No: 64-17-5), sodium carbonate (CAS No: 497-19-8), hydrochloric acid (CAS No: 7647-01-0), 2,4,6-tripyridyl-s-triazine – TPTZ (CAS No: 3682-35-7), 2M Folin-Ciocalteu`s phenol reagent were purchased from Sigma-Aldrich, USA. All chemicals and solvents were for HPLC grade.

2.3. Equipment

UV/VIS Spectrophotometer “Spectroquant Pharo 300” was equipped with a xenon flash lamp, a grating monochromator with a step motor with a wavelength range of 190 – 1000 nm ±1 nm accuracy. The photometric measurement was performed by a photodiode light sensor with a measuring range of A= -3.300 to A= +3.300 (Merck KGaA, Germany). Spectrophotometer was used for the determination of TPC and antioxidant activity by DPPH, ABTS, and FRAP methods.

An automatic autoanalyzer (SEAL Analytical - AA3, Germany) was used for the determination of the content of total alkaloids as nicotine by 460 nm. AA3 is equipped with a multi-speed peristaltic pump and a digital photometer (24-bit high-resolution A/D converter, linear range 0-1.8 (ABS)), detection resolution: 0.1 ug/L, autosampler, and AACE software package.

The ultrasonic bath “Elma Transsonic T 460/H” with a frequency of 460 H was used for ultrasonic extraction (Elma Schmidbauer GmbH, Germany).

2.4. Methods

2.4.1. *Preparation of tobacco seed extracts (TSE)*

Samples from grounded TWS were weighed (0.1 g) on an analytical balance and placed into conical flasks for extraction. The extraction process was performed with four types of solvent – H_2O, 99.9% CH_3OH, 60% CH_3OH, and 96% C_2H_5OH under different conditions – maceration for 24 h, ultrasound extraction for 20 min at 20^oC, and ultrasound extraction for 20 min at 40^oC. All solvents were used in a volume of 4 mL, 5 mL, and 6 mL. The proportion of the sample-solvent ratio was based on the previous research of the study group on the method for the extraction of polyphenols from tobacco leaves (Docheva *et al*., 2018). The extracts were filtered: An aliquot of the extracts obtained was subjected to analysis for total phenolic content using the Folin–Ciocalteu method and antioxidant activity using the DPPH free radical scavenging method (DPPH method), ABTS radical scavenging method (ABTS method), and Ferric reducing antioxidant power assay (FRAP method).

2.4.2. *Determination of the content of total alkaloids as nicotine - Continuous-flow analysis method*

An extract of the TWS was prepared with 5% acetic acid and the total alkaloids (as nicotine) content of the extract was determined by reaction with sulphanilic acid and cyanogen chloride. Cyanogen chloride was generated *in situ* by the reaction of potassium cyanide and chloramine - T. The color developed was measured at 460 nm (ISO 15152:2003).

2.4.3. *Determination of total phenolic content using the Folin-Ciocalteu method*

The amount of total phenols (TPC) was based on the Folin–Ciocalteu (FC) method (Singleton & Rossi, 1965) with some modification. 0.1 mL TSE, 6 mL H_2O, and 0.5 mL 0.2 M Folin–Ciocalteu reactive were placed into the test tube. After 4 min 3.4 mL 7.5% Na_2CO_3 was added. All the samples were stored in the dark for 2 hours and then were measured at 765 nm. The concentration of the phenolic compounds in the extracts was calculated using Gallic acid as standard, and the results were expressed as milligrams of Gallic acid equivalents per gram extract (mg GAE/g).

2.4.4. *DPPH free radical scavenging method (DPPH)*

The DPPH radical (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was carried out as reported by Docheva *et al*. (2014). A solution of $DPPH^{\bullet}$ reagent was prepared daily with a concentration of 0.12 mM (0.0048 g $DPPH^{\bullet}$ was dissolved in 100 mL CH_3OH). Two mL from the DPPH solution was placed in a vessel and 2 mL of TSE was added. The mixtures were placed in the dark for 30 min at room temperature. The absorbance was measured at 515 nm using a spectrophotometer. The results were presented as Trolox equivalents per gram extract (mM TE/g).

2.4.5. *ABTS radical scavenging method (ABTS)*

The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging activity was determined by the method previously reported by Kirkova *et al.* (2020). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reaction between 7 mM ABTS in water and 2.45 mM $K_2S_2O_8$ in water, stored in the dark at room temperature for 12–16 h. $ABTS^{\bullet+}$ solution was diluted with CH_3OH to an absorbance of 0.70±0.02 at 734 nm. The mixture of $ABTS^{\bullet+}$ and TSE (1:9 v/v) was placed in a vessel in the dark at room temperature and after 10 min absorbance was measured at 734 nm. The results were presented as Trolox equivalents per gram extract (mM TE/g).

2.4.6. *Ferric reducing antioxidant power method (FRAP)*

The method is based on the reduction of the ferric tripyridyl-s-triazine complex (Fe^{3+} TPTZ) to a ferro-form (Fe^{2+} TPTZ), which has an intense blue color with an absorption maximum of 593 nm. The FRAP assay was conducted according to the original method reported by Benzie and Strain (1966) and modified by Docheva *et al*. (2020). FRAP reagent was prepared by 0.3 M acetate buffer (pH 3.6), ferric chloride dissolved in water, and 0.0312 g TPTZ dissolved in 10 mL 0.04 M HCl mixing in ratio 10:1:1 v/v/v. For the assay 0.05 mL TSE, 0.15 mL distilled water, and 1.5 mL FRAP reagent were mixed. Blue color became visible and after 15 min in the dark, the absorbance of the sample was measured at 593 nm. The results were presented as Trolox equivalents per gram extract (mM TE/g).

2.4.7. *Calculation of relative antioxidant activity index (RACI)*

The RACI is the mean value of the standard scores transformed from the initial data generated by different methods, with no unit limitation and no variance between methods. The following equitation analyzed the relative antioxidant capacity index (RACI):

$$\text{RACI} = \frac{x - \mu}{\sigma}$$

Where x – represents the raw data, μ – the mean value, σ – the standard deviation of the assays (Gorjanovic´ *et al.,* 2013).

2.4.8. *Statistics*

All experimental procedures were done in triplicate. The quantitative data were expressed as mean ± standard deviation (SD). Samples were divided into three groups: maceration, ultrasound 20ºC, and ultrasound 40ºC while One-way ANOVA and Duncan`s test were employed to identify differences in the means of each group. Statistics were analyzed using

IBM SPSS Statistics (version 25, IBM Corp.) and significance was declared at $p<0.05$. Correlation was performed using MS Excel Correlation Data Analysis.

3. RESULTS

3.1. Total Phenolic Content (TPC)

The TPC in TSE, prepared by different solvents under maceration with different sample/solvent ratios, is presented in Table 1. The highest TPC was achieved in TSE with maceration, obtained by C_2H_5OH, ratio 0.1 g/6 mL (5.85±0.63 mg/g GAE) and followed by extract, obtained by 60% CH_3OH, ratio 0.1 g/5 mL (2.34±0.28 mg/g GAE). The lowest TPC was reported for extraction with C_2H_5OH, ratio 0.1 g/4 mL (0.80±0.11 mg/g GAE). There wasn`t a significant difference in the TPC of the TSE with CH_3OH between the sample/solvent ratios.

Under ultrasound extraction at 20°C for 20 min, the TPC varied between 0.96±0.09 mg/g GAE (0.1 g/4 mL H_2O) and 2.12±0.68 mg/g GAE (0.1 g/ 4 mL 60% CH_3OH). It is noteworthy that the TPC in TSE was higher when the extraction was in the ratio 0.1g/4 mL and 0.1g/5 mL solvent CH_3OH, 60% CH_3OH, and C_2H_5OH, compared to the sample/solvent ratio 0.1g/6 mL. The highest TPC in TSE obtained by ultrasound extraction at 40°C in sample/solvent ratio 0.1 g/5 mL 60% CH_3OH (2.71±1.06 mg/g GAE) and 0.1 g/4 mL 60% CH_3OH (2.62±0.55 mg/g GAE) was reported. There were differences in the TPC in TSE, obtained by ultrasonic extraction at 20°C and 40°C. TPC obtained with CH_3OH and 60% CH_3OH was higher as the temperature rose. Generally, 60% CH_3OH extracted the largest amount of total phenols in all the extraction methods described.

Table 1. Total phenolic content (TPC, mg/g GAE) in tobacco waste seed extracts obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1g/6 mL
TPC, mg/g GAE	Maceration/24 h	H_2O	0.98±0.32^{ab}	1.46±0.43^{bc}	0.94±0.03^{ab}
		CH_3OH	1.11±0.20^{abc}	1.36±0.26^{abc}	1.06±0.13^{abc}
		60% CH_3OH	1.12±0.35^{abc}	2.34±0.28^{d}	1.56±0.10^{c}
		C_2H_5OH	0.80±0.11^{a}	0.89±0.08^{ab}	5.85±0.63^{e}
	Ultrasound 20°C/20 min	H_2O	0.96±0.09^{a}	1.12±0.20^{a}	1.02±0.09^{a}
		CH_3OH	1.48±0.11^{abc}	1.72±0.34^{abc}	1.16±0.39^{ab}
		60% CH_3OH	2.12±0.68^{c}	1.32±0.26^{ab}	1.18±0.16^{ab}
		C_2H_5OH	1.96±0.56^{bc}	1.09±0.36^{a}	1.05±0.10^{a}
	Ultrasound 40°C/20 min	H_2O	1.00±0.09^{a}	1.31±0.49^{a}	1.19±0.04^{a}
		CH_3OH	1.14±0.24^{a}	1.39±0.23^{a}	2.02±1.05^{ab}
		60% CH_3OH	2.62±0.55^{b}	2.71±1.06^{b}	1.31±0.10^{a}
		C_2H_5OH	1.06±0.01^{a}	1.09±0.54^{a}	1.52±0.51^{a}

Note: ^{a}Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different ($p<0.05$), n = 3.

3.2. Antioxidant Activity (AO)

The AO activity of TWS extracts was determined using the DPPH-assay, ABTS-assay, and FRAP-assay.

3.2.1. DPPH assay

The antioxidant activity of the TSE was analyzed with the DPPH assay for the three of the solvents (60% CH_3OH, CH_3OH, and C_2H_5OH). The radical scavenging activity of extracts, obtained under maceration, varied between 1.86 ±0.12 mM TE/g (0.1 g/4 mL CH_3OH) and 13.94±0.30 mM TE/g (0.1 g/6 mL C_2H_5OH) (Table 2). The maceration method obtained the highest antioxidant activity with C_2H_5OH as a solvent. The results for methanolic extracts did

not depend on the sample/solvent ratio. This ratio affected the extracts obtained by 60% CH_3OH as a solvent.

Radical scavenging activity for the extracts, obtained by ultrasound at 20ºC, varied between 1.07±0.80 mM TE/g (0.1 g/4 mL CH_3OH) and 8.49±0.32 mM TE/g (0.1 g/6 mL 60% CH_3OH), while at 40ºC - between 1.25±0.10 mM TE/g (0.1 g/4 mL CH_3OH) and 10.83±0.44 mM TE/g (0.1 g/6 mL C_2H_5OH). The extracts, obtained with 60% CH_3OH and C_2H_5OH, had higher antioxidant activity, determined by the DPPH method, compared to the CH_3OH. Methanolic extracts showed between 3- and 4 times lower antioxidant activity (an average of 2.14 mM TE/g) compared to other extracts (an average of 9.22 mM TE/g). 60% CH_3OH used for extraction with ultrasound at 20ºC was the most effective based on the results. It is noteworthy that the radical-scavenging activity of extracts, obtained under maceration, had a higher activity than those obtained by ultrasound extraction despite the lower content of phenolic acids. The values for ultrasound extraction at 40ºC, 0.1g/6 mL sample/solvent ratio are higher than those for ultrasound extraction at 20ºC and are close to the obtained with maceration.

Table 2. Antioxidant activity of tobacco seed extracts, determined by the DPPH method, obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1g/6 mL
DPPH, mM TE/g	Maceration/24 h	CH_3OH	1.86±0.12^{a}	2.16±0.05^{a}	2.41±0.08^{a}
		60% CH_3OH	7.26±0.43^{c}	8.71±0.53^{d}	10.23±0.45^{e}
		C_2H_5OH	6.19±0.76^{b}	8.96±1.29^{d}	13.94±0.30^{f}
	Ultrasound 20°C/20 min	CH_3OH	1.07±0.80^{a}	2.40±0.87^{ab}	2.10±0.44^{ab}
		60% CH_3OH	6.09±0.83^{cd}	6.58±0.40^{d}	8.49±0.32^{e}
		C_2H_5OH	5.83±0.99^{cd}	4.87±1.03^{c}	2.7±0.83^{b}
	Ultrasound 40°C/20 min	CH_3OH	1.25±0.10^{a}	2.01±0.45^{ab}	2.11±0.04^{ab}
		60% CH_3OH	3.92±0.89^{ab}	5.64±0.40^{b}	9.48±0.99^{c}
		C_2H_5OH	3.39±0.26^{ab}	5.33±0.57^{b}	10.83±0.44^{c}

Note: [a]Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different ($p<0.05$), n = 3.

3.2.2. *ABTS assay*

Another stable free radical cation, ABTS, was used to evaluate the antioxidant activity of TSE. The results were analyzed based on the influence of the extraction method on the same principle as the DPPH assay. The highest antioxidant activity under maceration, determined by ABTS assay, was reported in the extracts, obtained by H_2O and 60% CH_3OH (Table 3). The antioxidant activity in H_2O TSE varied between 3.82±2.85 mM TE/g (0.1 g/5 mL) and 6.16±1.55 mM TE/g (0.1 g/6 mL), while in 60% CH_3OH extracts it varied between 5.13±0.44 mM TE/g (0.1 g/6 mL) and 5.97±0.42 mM TE/g (0.1 g/5 mL). The antioxidant activity for ethanolic and methanolic extracts was less than 1.22±0.37 mM TE/g as the lowest antioxidant activity was reported in ethanolic extracts.

Antioxidant activity of TSE, obtained by ultrasound extraction at 20ºC and 40ºC for 20 min, varied between 0.98±0.47 mM TE/g 0.1g/4 mL CH_3OH and 6.03±1.16 mMTE/g 0.1g/4 mL 60% CH_3OH (Table 3). The antioxidant activity with ultrasound 20ºC performed with 60% CH_3OH depends on the sample/solvent ratio, and the highest result was achieved with 0.1g/4 mL. The ultrasound extraction with H_2O did not depend on the temperature and the results were close to high, between 4.32±0.36 and 5.57±1.29 mM TE/g for ultrasound 20ºC and 4.89±0.34 - 5.01±0.04 mM TE/g for ultrasound 40ºC.

The highest antioxidant activity, determined by the ABTS method by maceration and ultrasonic extraction, was reported in the extraction with 60% CH_3OH and H_2O, and lower for CH_3OH and C_2H_5OH (Table 3).

Table 3. Antioxidant activity of tobacco seed extracts, determined by ABTS method, obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1/6 mL
ABTS, mM TE/g	Maceration/24 h	H_2O	5.21±0.53^{bc}	3.82±2.85^{b}	6.16±1.55^{c}
		CH_3OH	1.13±0.22^{a}	1.22±0.37^{a}	1.15±0.15^{a}
		60% CH_3OH	5.17±0.61^{bc}	5.97±0.42^{c}	5.13±0.44^{bc}
		C_2H_5OH	1.22±0.62^{a}	0.20±0.32^{a}	0.15±0.29^{a}
	Ultrasound 20°C/20 min	H_2O	4.85±0.28^{cd}	5.57±1.29^{cd}	4.32±0.36^{cd}
		CH_3OH	0.98±0.47^{a}	1.43±1.42^{ab}	1.15±0.48^{a}
		60% CH_3OH	6.03±1.16^{d}	5.15±0.88^{cd}	3.84±0.37^{bcd}
		C_2H_5OH	1.46±0.60^{ab}	3.32±0.25^{abc}	1.41±1.16^{ab}
	Ultrasound 40°C/20 min	H_2O	4.89±0.34^{bc}	5.01±0.04^{bc}	4.95±2.05^{bc}
		CH_3OH	1.59±0.42^{a}	1.02±0.42^{a}	1.27±0.37^{a}
		60% CH_3OH	5.99±1.18^{c}	4.96±0.26^{bc}	5.45±1.30^{c}
		C_2H_5OH	3.13±2.32^{ab}	1.32±0.59^{a}	1.68±1.45^{a}

Note: [a]Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different (p<0.05), n = 3.

3.2.3. *FRAP assay*

The antioxidant activity of TSE, obtained by different sample/solvent ratios (H_2O, CH_3OH, 60% CH_3OH, and C_2H_5OH) under different extraction conditions by the FRAP method, is presented in Table 4. The antioxidant activity determined by FRAP-assay varied between 2.07±0.14 mM TE/g, (0.1g/4 mL C_2H_5OH) by maceration and 7.60±0.53 mM TE/g (0.1g/4 mL 60% CH_3OH) by ultrasound extraction at 40°C. The extracts, obtained upon maceration and ultrasound extraction by CH_3OH and 60% CH_3OH, had slightly higher antioxidant activity, compared to the C_2H_5OH and H_2O extracts (Table 4). Solvent/sample ratios 0.1 g/4 mL and 0.1 g/5 mL ultrasound extraction at 20°C and 40°C represent better results with CH_3OH and 60% CH_3OH than 0.1 g/6 mL. The results obtained with H_2O by maceration represent significantly different results in 0.1 g/6 mL sample/solvent ratio, close to that obtained with ultrasound extraction at 20°C.

Table 4. Antioxidant activity of tobacco seed extracts, determined by method, obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1g/6 mL
FRAP, mM TE/g	Maceration/24h	H_2O	2.98±0.36^{abc}	3.14±0.25^{abc}	4.58±1.67^{bcd}
		CH_3OH	5.13±2.24^{cd}	4.41±0.63^{abcd}	6.26±3.10^{de}
		60% CH_3OH	7.49±1.22^{e}	7.53±1.65^{e}	5.08±0.68^{cd}
		C_2H_5OH	2.07±0.14^{a}	2.41±0.22^{ab}	2.46±0.11^{ab}
	Ultrasound 20°C/20 min	H_2O	3.33±0.40^{a}	4.43±0.72^{abcd}	4.33±0.07^{abcd}
		CH_3OH	5.93±1.81^{cd}	5.69±0.88^{bcd}	3.99±1.35^{ab}
		60% CH_3OH	5.46±0.27^{bcd}	6.02±1.78^{d}	4.47±0.14^{abcd}
		C_2H_5OH	4.14±0.53^{abc}	3.07±0.16^{a}	3.34±0.14^{a}
	Ultrasound 40°C/20 min	H_2O	3.61±0.09^{a}	3.96±0.17^{ab}	3.65±0.83^{a}
		CH_3OH	6.60±0.48^{bc}	5.48±2.36^{b}	5.26±2.66^{b}
		60% CH_3OH	7.60±0.53^{c}	7.19±1.39^{c}	5.07±0.82^{b}
		C_2H_5OH	3.95±1.01^{ab}	4.98±0.31^{b}	4.73±1.37^{b}

Note: [a]Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different (p<0.05), n = 3.

3.3. Correlation Between TPC and AO Activity

The correlation between TPC and the antioxidant activity of extracts, obtained by the same extragents, is presented in Table 5. Table 5 shows that there were weak correlations between

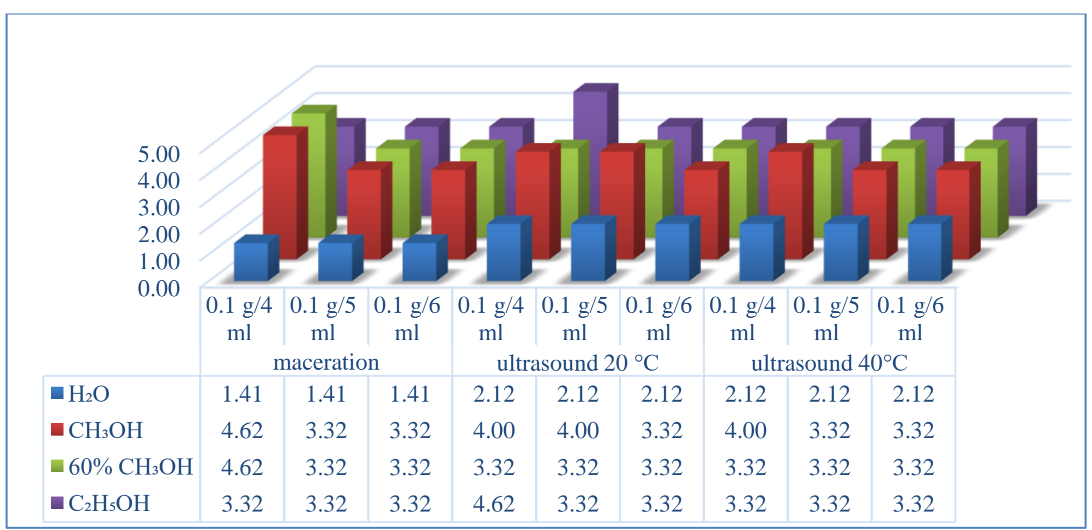
TPC and antioxidant activity. The highest correlation between DPPH assay and TPC was reported in extracts, obtained by C_2H_5OH – R^2=0.5468, followed by DPPH assay and TPC was reported in extracts, obtained by 60 % CH_3OH - R^2=0.3409. The correlation between the antioxidant activity by ABTS-assay and the TPC was not observed. The highest correlation was established in H_2O extracts (R^2=0.3124). The correlation between FRAP-assay and TPC was not observed, similar to the DPPH and ABTS-assay. R^2 varied between R^2=0.0021 (CH_3OH) and R^2=0.3238 (60% CH_3OH).

Table 5. Linear regression between total phenolic content (mg/g GAE) and antioxidant capacity by DPPH assay, ABTS assay, and FRAP assay.

Method	Solvent			
	H_2O	CH_3OH	60% CH_3OH	C_2H_5OH
DPPH	-	0.0223	0.3409	0.5468
ABTS	0.3124	0.0106	0.2863	0.2193
FRAP	0.0505	0.0021	0.3238	0.1743

3.4. Evaluation of Relative Antioxidant Capacity Index (RACI)

To achieve a more reliable comparison between the TSE and Relative Antioxidant Capacity Index (RACI) was determined. As can be seen in Figure 1, the highest values for RACI (4.62) were observed in the extracts, obtained by maceration 0.1g /4 mL CH_3OH and 60% CH_3OH and by ultrasound extraction at 20°C, 0.1g /4 mL CH_3OH. Aqueous extracts had the lowest RACI values because they were studied only by the ABTS and FRAP assays. For extraction under maceration, the RACI value for water extracts was 1.41, while extraction by ultrasound was twice as high – 2.12. The remaining extracts occupy intermediate values of the RACI value – 3.32. It is noteworthy that there was no difference in the RACI coefficient, or in the total antioxidant activity of the extracts obtained by the different extraction methods.



	0.1 g/4 ml	0.1 g/5 ml	0.1 g/6 ml	0.1 g/4 ml	0.1 g/5 ml	0.1 g/6 ml	0.1 g/4 ml	0.1 g/5 ml	0.1 g/6 ml
	maceration			ultrasound 20 °C			ultrasound 40°C		
H_2O	1.41	1.41	1.41	2.12	2.12	2.12	2.12	2.12	2.12
CH_3OH	4.62	3.32	3.32	4.00	4.00	3.32	4.00	3.32	3.32
60% CH_3OH	4.62	3.32	3.32	3.32	3.32	3.32	3.32	3.32	3.32
C_2H_5OH	3.32	3.32	3.32	4.62	3.32	3.32	3.32	3.32	3.32

Figure 1. Relative antioxidant capacity index (RACI) of tobacco seed extract.

4. DISCUSSION and CONCLUSION

The results of the study expressed data on the total phenolic content of the TWS and the antioxidant activity of the TSE obtained from the TWS. The Post Host Test (Duncan`s test) was provided to check the differences between the mean values obtained for each of the analyzed extracts in the group and to determine the statistical similarity between them. The expected result is organic production to imply higher levels of synthesized secondary metabolites such as polyphenolic compounds as it is based on the biological defense of plants against harmful environmental factors. Phenolic acids are health-beneficial compounds, bioactive components

that are capable of delaying or inhibiting the oxidation processes that occur under the influence of atmospheric oxygen or reactive oxygen species (Rodgman & Perfetti, 2013). The phenolic acids of plant extracts can be determined as a total phenolic content (TPC). Xie *et al.* (2011) reported the total phenolic content of tobacco seeds to be 1.24 – 2.26 mg GAE/g when polyphenols were extracted with acetone and 1.20 – 1.44 mg GAE/g using 80% ethanol. The solvents used in the present scientific study showed better results than those reported in the literature (Table 1). The higher results obtained for TPC could be a result of the organic production of the tobacco seeds, considering that polyphenol synthesis depends on the growing conditions and is a response to the abiotic and biotic stresses (Corso *et al.*, 2020). Generally, 60% CH_3OH extracted the higher amount of total phenols in all the three described extraction methods in the study. The results agree with other investigators, which concluded that the highest yield of phenolics from different tobacco plants was observed with extraction with 60% CH_3OH (Dagnon & Edreva, 2003; Docheva *et al.,* 2014; Sun *et al*., 2015). On the other hand, the content of phenolic acids in Bulgarian oriental tobaccos Krumovgrad 988 and Krumovgrad 90 were 6.38 mg/g and 8.04 mg/g respectively (Docheva *et al.,* 2014). Comparing the content of TPC in tobacco leaf extracts with that in TSE, it can be concluded that tobacco seed extracts have 5 – 6 times lower content of phenolic compounds. The TSE was also analyzed for nicotine content. The result was negative, which is an advantage over the extracts obtained from the leaves. There is a similarity between the values obtained for TPC in TSE to that of aqueous extracts, obtained from medicinal plants. Research on water extracts of seven medicinal plants used in phytotherapy (wild yams, dandelion, leuzea, asparagus, Benedectine thistle, cotton thistle, and sarsaparilla) shows that the total phenols were between 0.87 mg GAE/g and 7.16 mg GAE/g (Angelova & Petkova, 2019). Tobacco plants are also known as medical plants and further analyses for individual polyphenol composition will be needed for better comparison.

The antioxidant activity of the TSE was analyzed with three methods, namely DPPH, ABTS, and FRAP methods. These methods were chosen, because they are rapid, robust, and accurate for systematically assessing the total antioxidant capacity of extracts from plant materials on a large scale (Piluzza & Bullitta, 2011). The combination of the DPPH and ABTS methods allows a more complete characterization of the antioxidant properties of the TSE due to the different mechanisms of elimination of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, while the FRAP method determines the ability of the substances in the TSE to reduce Fe^{+3} to Fe^{+2}.

The highest antioxidant activity was recorded in the DPPH method, followed by the ABTS-method and the FRAP method. The DPPH assay is one of the widely used methods and, together with the ABTS assay, is commonly used to measure the total antioxidant activity of various biological samples by measuring radical scavenging through electron donation (Birasuren *et al.,* 2013). The results for DPPH and ABTS methods (Tables 2 and 3) agreed with the data reported by Xie *et al.* (2011), who reported the antioxidant activity of extracts from tobacco seed flour from Maryland variety by the following methods: DPPH radical scavenging capacity (RDSC) of extracts of seed flour 2.65 – 4.27 µmol Te/g; radical cation ABTS•+ scavenging capacity – 3.42 – 6.89 µmol Te/g; and oxygen radical absorbance capacity (ORAC) – 44 – 74 µmol Te/g. Comparability was observed between antioxidant activity in the extracts, investigated by the DPPH method and the ABTS method with CH_3OH solvent. The antioxidant activity of the C_2H_5OH extracts and 60% CH_3OH extracts was shown to be lower in the ABTS method compared to the DPPH method. Sargi *et al*. (2013) reported similar relation that the extracts of golden flax (3.38 ±0.09 Mmol TEAC g^{-1}), brown flax (3.70 ±0.09 Mmol TEAC g^{-1}) had higher antioxidant activity (ABTS assay) than the results obtained with the same extracts with the DPPH method (golden flax 1.16 ±0.04 Mmol TEAC g^{-1}; brown flax 1.56 ±0.01 Mmol TEAC g^{-1}).

FRAP assay is commonly used to study the antioxidant capacity of plant materials. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The simple and reliable FRAP assay measures the reducing potential of an

antioxidant reacting with a ferric-TPTZ (Fe(III)-TPTZ) complex and producing a colored ferrous-TPTZ (Fe(II)-TPTZ) complex by a reductant at a low pH (Munteanu & Apetrei, 2021). The extracts, obtained by maceration with CH_3OH and C_2H_5OH, had higher antioxidant activity with the FRAP assay compared to the ABTS assay, while ethanolic extracts had lower antioxidant activity compared to the DPPH assay. The antioxidant activity of TSE is higher compared to the extracts of seeds, rich in Omega-3 – Chia (2.86 ± 0.10 Mmol TEAC g^{-1}), golden flax (0.33 ± 0.10 Mmol TEAC g^{-1}), brown flax (0.76 ± 0.01 Mmol TEAC g^{-1}), white perilla (4.01 ± 0.29 Mmol TEAC g^{-1}), and brown perilla (5.24± 0.09 Mmol TEAC g^{-1}), investigated by FRAP-assay (Sargi *et al*., 2013).

The results obtained for the correlation between TPC and AO activity (Table 5) can be discussed with different qualitative and quantitative compositions in the extracts that suppress or enhance the antioxidant activity. The results obtained by Docheva *et al.* (2020) for tobacco extracts are similar; the researcher concluded that there were some other chemical components in the tobacco extracts, other than phenolics, which were variety-dependent and influenced the DPPH radical scavenging activity. Youn *et al*. (2019) established a weaker correlation between the FRAP value and the concentrations of TPC in extracts, obtained from *Dendropanax morbifera LEV.,* while Angelova and Petkova (2019) found a positive linear correlation between TPC and antioxidant activity (DPPH and FRAP) – R> 0.9 of 7 extracts of medicinal plants.

Figure 1 reveals that the RACI values of all TSE are positive, which indicates that the extracts have a high relative antioxidant capacity. The results for RACI value in the present study are higher than those reported for nettle leaf extracts - RACI value between −1.589 and +1.108 reported (Knežević *et al.,* 2019).

A comparison between the AO activity of the TSE and extracts from tobacco leaves shows that the antioxidant activity of TSE was almost 10 times lower. Docheva *et al.* (2020) established that the antioxidant activity of tobacco extracts, obtained by 60% CH_3OH varied between 76.50±7.12 $mMFe^{2+}$/g (Myumunovo seme) and 46.66±4.23 $mMFe^{2+}$/g (Djebel basma 1). The difference in antioxidant activity between ethanolic seed extracts and ethanolic leaf tobacco extracts (between 7.89±0.85 $mMFe^{2+}$/g DM – Djebel basma 1 and 17.14±1.72 $mMFe^{2+}$/g DM Myumunovo seme) was even more than 10 times greater.

The total result for antioxidant activity is a prerequisite for further studies using other methods. The paucity of published information on the content of polyphenols in tobacco seeds and their antioxidant activity gives scope for future scientific research in the field of utilization of this type of source. In conclusion, the study extended the information on the accumulation of phenolic compounds in tobacco seeds and the antioxidant activity of extracts derived from them. Maceration with 60% CH_3OH as a solvent could be an appropriate method for obtaining polyphenolic extracts from tobacco waste seeds, with antioxidant activity. Further studies will be necessary to expand the knowledge of the influence of organic production on the accumulation of polyphenolic compounds. Based on the research carried out, tobacco seeds offer the opportunity to obtain extracts of polyphenolic compounds suitable for potential applications in the pharmaceutical and food industries and the possibility of utilizing tobacco waste seeds.

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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

Liliya Stoyanova: Fundings, Conception, Materials, Data collection and processing, Analysis and Interpretation. **Maria Angelova-Romova**: Analysis and Interpretation, Supervision, and Writing **Margarita Docheva**: Design, Literature review. **Desislava Kirkova**: Design, Supervision.

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REFERENCES

Abbas Ali, M., Abu Sayeed, M., Kumar Roy, R., Yeasmin, S., Mohal Khan, A. (2008). Comparative Study on Characteristics of Seed Oils and Nutritional Composition of Seeds from Different Varieties of Tobacco (*Nicotiana tabacum* L.) Cultivated in Bangladesh. *Asian Journal of Biochemistry*, *3*, 203-212. https://doi.org/10.3923/ajb.2008.203.212

Alara, O., Abdurahman, N., Ukaegbu, C. (2021). Extraction of phenolic compounds: A review. *Current Research in Food Science*, *4*, 200-214. https://doi.org/10.1016/j.crfs.2021.03.011

Angelova, E., Petkova, N. (2019). Determination of biologically active substances in extracts of seven medicinal plants. *Youth forums "Science, technology, innovation, business - 2019"*, 66-71.

Banozic, M., Babic, J., Jokic, S. (2020). Recent advances in extraction of bioactive compounds from tobacco industrial waste-a review. *Industrial Crop and Production,* 112009, 144. https://doi.org/10.1016/j.indcrop.2019.112009

Benzie, I., Strain, J. (1966). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*; *239*, 70-76. https://doi.org/10.1006/abio.1996.0292

Birasuren, B., Kim, N.Y., Jeon, H.L., Kim, M.R. (2013). Evaluation of the antioxidant capacity and phenolic content of *Agriophyllum pungens* seed extracts from Mongolia. *Preventive Nutrition and Food Science*, *18*(3), 188-195. https://doi.org/10.3746/pnf.2013.18.3.188

Bozukov, H. (2018). Biological/organic production of tobacco. *Bulgarian Tobacco*, *1-2*, 8-12.

Bozukov, H., Kasheva, M., Kochev, Y., & Vitanova, D. (2019). Evaluation of oriental tobacco of the variety group of Basmi upon organic production. *Bulgarian Journal of Agricultural Science, 25*(4), 633-637.

Corso, M., Perreau, F., Mouille, G., Lepiniec, L. (2020). Specialized phenolic compounds in seeds: structures, functions, and regulations. *Plant Science, 296*, 110471. https://doi.org/10.1016/j.plantsci.2020.110471

Dagnon, S., Edreva, A. (2003). Application of pattern recognition method for color assessment of oriental tobacco based on HPLC of polyphenols. *Contributions to Tobacco Research, 20*(5), 355-359.

Docheva, M.H., Kochev, Y.G., Kirkova, D.M., Stoilova, A.B. (2020). Antioxidant activity and chemical composition of crude extracts from different tobaccos and tobacco blends. *Bulgarian Chemical Communications, 52*(D), 149-154.

Docheva, M.H., Popova, V.T., Ivanova, T.A., Nikolova, V.V., Hristeva, T.H., Nikolov, N.N. (2018). Polyphenol content and antioxidant activity of aqueous/methanol extracts from different tobacco species (*Nicotiana*). *Bulgarian Chemical Communications*, *50*(4), 553-559.

Docheva, M., Dagnon, S., Statkova-Abeghe, S. (2014). Flavonoid content and radical scavenging potential of extracts prepared from tobacco cultivars and waste. *Natural Product Research, 28*(17), 1328-1334. http://dx.doi.org/10.1080/14786419.2014.902947

Frega, N., Bocci, F., Conte, L.S., Testa, F. (1991). Chemical composition of tobacco seeds (*Nicotiana tabacum* L.). *Journal of the American Oil Chemists' Society*, *68*(1), 29-33. https://doi.org/10.1007/BF02660305

Gorjanovic´, S., Alvarez-Suarez, J., Novakovic´, M., Lato Pezo, F., Battino, M., Suzˇnjevic´, D. (2013). Comparative analysis of antioxidant activity of honey of different floral sources using recently developed polarographic and various spectrophotometric assays. *Journal of Food Composition and Analysis, 30*, 13-18. http://dx.doi.org/10.1016/j.jfca.2012.12.004

ISO 15152 (2003). Determination of the content of total alkaloids as nicotine Continuous-flow analysis method.

Kirkova, D., Statkova-Abeghe, S., Docheva, M., Stremski, Y., Minkova, S. (2020). Structure-activity relationship of in vitro radical-scavenging activity of 2-(hydroxyphenyl) benzothiazole derivatives. *Bulgarian Chemical Communications, 52*(D), 196-200.

Knežević, V., Pezo, L., Lončar, B., Filipović, V., Nićetin, M., Gorjanović, S., Šuput, D. (2019). Antioxidant Capacity of Nettle Leaves During Osmotic Treatment. *Periodica Polytechnica Chemical Engineering*, *63*(3), 491-498. https://doi.org/10.3311/PPch.12688

Kochev, Y. (2008). Investigation of the influence of the parameters in the separation of tobacco seeds in an inclined air duct. *"USB-Plovdiv, Jubilee Scientific Session" 60 Years of USB - Plovdiv, Series C, Natural and Human Sciences, VII*, 119-122.

Kumar, A., Nirmal, P., Kumar, M., Jose, A., Tomer, V., Oz, E., Proestos, C., Zeng, M., Elobeid, T., Sneha, K., Oz, F. (2023). Major Phytochemicals: Recent Advances in Health Benefits and Extraction Method. *Molecules*, *28*(2), 1-41. https://doi.org/10.3390/molecules28020887

Munteanu, I., Apetrei, C. (2021). Analytical methods used in determining antioxidant activity: A review. *International Journal of Molecular Sciences, 22*(7), 3380. https://doi.org/10.3390/ijms22073380

Özcan, M., Uslu, N., Lemiasheuski, V., Kulluk, D., Gezgin, S. (2023). Effect of roasting on the physico-chemical properties, fatty acids, polyphenols and mineral contents of tobacco (*Nicotiana tabacum* L.) seed and oils. *Journal of the American Oil Chemists' Society*, *100*(5), 403-412. https://doi.org/10.1002/aocs.12680

Piluzza, G., Bullitta, S. (2011). Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. *Pharmaceutical Biology, 49*(3), 240-247. https://doi.org/10.3109/13880209.2010.501083

Popova, V., Petkova, Z., Ivanova, T., Stoyanova, M., Lazarov, L., Stoyanova, A., Hristeva, T., Docheva, M., Nikolova, V., Nikolov, N., Zheljazkov, V. (2018). Biologically active components in seeds of three *Nicotiana* species. *Industrial Crops & Products, 117*, 375-381. https://doi.org/10.1016/j.indcrop.2018.03.020

Raei, Y., Aghaei-Gharachorlou, P. (2015). Organic cultivation of industrial crops: A review. *Journal of Biodiversity and Environmental Sciences*, *6*(1), 336-377.

Rodgman, A., Perfetti, T.A. (2013). *The Chemical Components of Tobacco and Tobacco Smoke*, 2nd ed. *CRC Press*.

Rossi, L., Fusi, E., Baldi, G., Fogher, C., Cheli, F., Baldi A., Dell`Orto. V. (2013). Tobacco Seeds By-Product as Protein Source for Piglets. *Open Journal of Veterinary Medicine, 3*(1), 73-78. https://doi.org/10.4236/ojvm.2013.31012

Sargi, S.C., Silva, B.C., Santos, H.M.C., Montanher, P.F., Boeing, J.S., Santos Júnior, O.O., Souza, N.E., Visentainer, J.V. (2013). Antioxidant capacity and chemical composition in seeds rich in omega-3: Chia, flax, and perilla. *Food Science and Technology, 33*(3), 541-548. https://doi.org/10.1590/S0101-20612013005000057

Singleton, V.L., Rossi, J.A. (1965). Colorimetry of total phenolic with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144-58.

Sun, P., Liu, Y., Yi, Y., Li, H., Fan, P., Xia, C. (2015). Preliminary enrichment and separation of chlorogenic acid from *Helianthus tuberosus* L. leaves extract by macroporous resins. *Food Chemistry, 168*, 55-62. http://dx.doi.org/10.1016/j.foodchem.2014.07.038

Tomov, N., Chotova, T., Apostolov, N. (1983). Influence of tobacco seed size on their sowing qualities. *Agriculture science, 22*(2), 59-66.

Usta, N. (2005). Use of tobacco seed oil methyl ester in a turbocharged indirect injection diesel engine. *Biomass Bioenergy*, *28*(1), 77-86. https://doi.org/10.1016/j.biombioe.2004.06.004

Usta, N., Aydoğan, B., On, A.H., Uğuzdǒan, E., Özkal, S.G. (2011). Properties and quality verification of biodiesel produced from tobacco seed oil. *Energy Convers. Manag.*, *52*(5), 2031-2039. https://doi.org/10.1016/j.enconman.2010.12.021

Xie, Z., Whent, M., Lutterodt, H., Niu, Y., Slavin, M., Kratochvil, R., Yu, L. (2011). Phytochemical, antioxidant, and antiproliferative properties of seed oil and flour extracts of Maryland-grown tobacco cultivars. *Journal of Agricultural and Food Chemistry, 59*(18), 9877-9884. https://doi.org/10.1021/jf202069g

Youn, J.S., Kim, Y.J., Na, H.J., Jung, H.R., Song, C.K., Kang, S.Y., Kim, J.Y. (2019). Antioxidant activity and contents of leaf extracts obtained from *Dendropanax morbifera* LEV are dependent on the collecting season and extraction conditions. *Food Science and Biotechnology, 28*(1), 201-207. https://doi.org/10.1007/s10068-018-0352-y

Zlatanov, M., Angelova, M., Antova, G., (2007). Lipid Composition of Tobacco Seeds, *Bulgarian Journal of Agricultural Science, 13*(5), 539-544.

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journal homepage: https://dergipark.org.tr/en/pub/ijsm **Research Article**

Biological control of pathogenic fungi using *Pseudomonas brassicacearum* isolated from *Aronia × prunifolia* (Marshall) Rehder roots

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Abstract: Endophytic bacteria, which are the subject of this study, serve as natural antifungal agents in the struggle against fungal infections, offering an eco-friendly alternative to chemical fungicides. So, it was aimed to determine the antifungal capacities of endophytic bacteria from *Aronia ×prunifolia* roots in the study. 25 endophytic bacteria were isolated, and their ability to act as biocontrol agents was evaluated by measuring fungal growth inhibition and chemical properties. Later, bacteria that showed a positive effect were identified through 16S gene sequencing. The results showed that the LB2 bacteria had the greatest ability to inhibit the selected fungi and the biochemical tests showed that the bacteria were Gram-negative, did not form spores, their colonies were well defined, and they could break down starch and gelatin, which was later diagnosed as *Pseudomonas brassicacearum* according to phylogenetic relationships. This study is the first report on which *P. brassicacearum* was isolated from *A. ×prunifolia* roots for the first time. These findings contribute to our understanding of the potential of endophytic bacteria, particularly *P. brassicacearum*, as natural antifungal agents in plant and human protection, offering a promising and sustainable approach to combat fungal infections while reducing the use of chemical fungicides.

1. INTRODUCTION

The fungal kingdom is believed to have 1.5 million species on our planet; of around 100,000 known fungal species, 400 have been recognized as pathogens to humans, animals and plants. Synthetic substances, such as antifungal medicines and fungicides, are commonly used to avoid the detrimental effects of fungus on human health and agriculture (Alsohiby *et al*., 2016; Yang *et al*., 2016). Isolating novel molecules from biological resources has attracted much interest due to the demand for safe and efficient antifungal treatments.

To maximize the likelihood of finding new antifungals, there is a continuous global search for new bacterial populations since the synthesis of antifungal metabolites in bacteria is highly dependent on strain and species. Recently, the use of microorganisms as a source of bioactive chemicals has attracted the attention of researchers (Anand *et al*., 2023). In environments that support the growth of a mixed bacterial and fungus flora, antifungal activity is a relatively

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common characteristic of bacteria that offers an ecological benefit (Santra & Banerjee, 2023). Endophytic bacteria can be isolated from plant tissues infected on their surface or from inside plants without endangering the host plants (Naranjo *et al*., 2023).

The potential of endophytic bacteria as biological control agents is quite promising. Berg and Hallmann (2006) found that a significant portion of the naturally occurring endophytic bacteria in plant roots had the ability to fight against fungal infections. This finding has implications for developing therapeutic antifungal drugs and creating plant protection chemicals (Boonman *et al*., 2023). A single plant may have many distinct bacterial endophyte species (Zinniel *et al*., 2002). Recent studies on plant endophytic bacteria have focused on their roles in plant nutrition (Cipriano *et al*., 2021; Rana *et al*., 2021; Adeleke & Babalola, 2022); pollutant catabolism (Siciliano *et al*., 2001); stress or defense responses (Cho *et al*., 2002); and invading pathogens (Sturz, 1999). Many endophytes have antifungal activity against fungi such as *Fusarium oxysporum* and *Rhizoctonia solani* on cotton (Chen *et al*., 1995), *Verticillium dahliae* and *Rhizoctonia solani* on potato (Berg *et al*., 2005), *Sclerotium rolfsii* on beans (Mahaffee & Kloepper, 1997), *Verticillium longisporum* on *Brassica napus (*Granér *et al*., 2003), and *Rhizoctonia solani*, *Fusarium oxysporum*, and *Phythium ultimunn* in balloon flower (Cho *et al*., 2002).

Aronia ×prunifolia (purple chokeberry) is native to North America and may be grown well in Europe and Asia (Szopa *et al*., 2017; Kulling & Rawel, 2008). Thus far, fruit extracts have been the subject of phytochemical studies that have revealed the presence of flavonoids, anthocyanins, proanthocyanidins, polyphenols, and hydroxycinnamic acids (Celka & Szkudlarz, 2010; Taheri *et al*., 2013; Szopa *et al*., 2017). Endophytic gram-negative bacteria like *Pseudomonas* sp. have been extensively studied as biological control agents in terms of the synthesis of antibiotic metabolites (Nielsen *et al*., 2002). Endophytic pseudomonads quickly and violently infiltrate the root system, preventing harmful bacteria from growing, promoting plant development, and increasing agricultural output (Singh *et al*., 2021).

This is the first report to determine the capacity of endophytic bacteria from *A. ×prunifolia* root to suppress pathogenic fungi. This study aimed to assess the antagonistic endophyte *Pseudomonas brassicacearum*'s capacity to control and produce bioactive extracellular compounds against the phytopathogenic *R. solani* and the two clinically important *T. rubrum* and *F. solani*.

2. MATERIAL and METHODS

2.1. Plant Material

The *A. ×prunifolia* plant was obtained from the Department of Biology, Faculty of Science, Ondokuz Mayis University, Samsun, Turkey.

2.2. Fungal Culture and Growth Maintenance

The virulent strains of *R. solani*, *T. rubrum* and *F. solani* responsible for human and plant diseases, were used. The strains were obtained from Ondokuz Mayis University, Department of Biology, Samsun, Türkiye. On potato dextrose agar (PDA) plates, the fungi were cultivated and incubated at 27 ± 2 °C for five days. For later usage, the fungal cultures were kept in PDA slants at 4 °C.

2.3. Endophytic Bacterial Isolation, Purification, and Culturing

To eliminate soil and dust, the root samples were washed in running tap water and then washed again with double-distilled water before processing. Surface sterilization was then carried out using laminar airflow. Surface sterilization is a vital step for getting rid of surface germs. Plant tissue was washed with 70% ethanol for 5 minutes, then with 2% sodium hypochlorite for 10 minutes, and then three times with sterile water. After being cleaned, plant samples were immersed for 15 minutes in a 10% sodium hydrogen carbonate ($NaHCO_3$) solution to disrupt and prevent endophytic fungal development (Cao *et al*., 2005). Afterward, tiny pieces of root

(0.5-1.0 cm) were cut, dried aseptically, and then plated on Nutrient agar (NA) which was then incubated at 27 ± 2 °C for 48 h for maximum recovery of bacterial colonies. Uncut, surface-disinfected root sections and uninfected parts were used as controls and incubated on the same medium. There was no growth on the control plate, there was no epiphytic contamination. To obtain bacterial isolates, physically different bacterial colonies on agar plates were chosen and repeatedly streaked after 48 hours. All purified bacterial isolates were kept at 4 °C after being subcultured on NA slants.

2.4. Phenotypic Identification

The biochemical and physiological characterization of selected bacteria was done using Berge's Manual of Systematic Bacteriology (Safaa & Qaysi, 2016). In addition to biochemical tests, phenotypic characteristics such as form, size, margin, surface, elevation, color, pigmentation, and Gram staining were used to define bacterial isolates. Known biochemical and physiological procedures, including the catalase test (3% H_2O_2), the oxidase reaction (Kovacs method), and the production of diffusible pigments, were used for the latter. Starch hydrolysis was finished by culture isolation on Starch nitrate agar media, and carbohydrate fermentation was carried out, in addition, using a medium containing specific carbohydrate sources (sucrose, glucose, lactose, mannitol, maltose, and rhamnose). After 24 hours of incubation at 37 °C, the plate was covered with iodine, and a definite zone enclosing the growth of colonies on the medium plate was found. When performing gelatin hydrolysis utilizing nutritional agar puncture tubes, liquefaction of the gelatin was seen after being inoculated with test bacteria (Sneath, 1992).

2.5. Molecular Identification

With slight modifications, Pascual's (2000) CTAB (cetyltrimethyl ammonium bromide) technique was used to extract DNA from fungal mycelia. The isolated samples' bacterial DNA was amplified using universal primers for 16S rDNA. According to Heuer *et al.* (1997), the study's primer sequences for amplification of the 16S rDNA gene were 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′). PCR amplification reactions were performed in a 50 µL reaction containing 1µL genomic DNA (1 ng µL-1), 1 µL (2.5 mM) dNTP mix (Sigma), 0.25 µL Taq DNA polymerase (5 U/µL) (Promega, Go-TaqFlexi DNA Polymerase), 1 µL each of primers (25 pmoles), 10 µL 5 × PCR buffer supplied by manufacturer (Promega, Go-Taq Green Buffer) and 3 µL MgCl2 (1.5 mM) (Sigma) and 32.75µL sterile ddH20. An initial denaturation step of 94 °C for 3 min was followed by 30 cycles of 94 °C for 1 min, 49 °C for 2 min, 72 °C for 3 min, and finally an extension step of 72 °C for 7 min during the PCR amplification (Salazar *et al*., 1999).

The 16S rDNA region's PCR products were sequenced by Macrogen (Macrogen Inc., Seoul, Republic of Korea). The program BioEdit version 7.2.5 was used to create a consensus sequence (Hall, 1999). Using the BLAST program, the consensus sequences for the 16S rDNA region were compared to the sequence data in GenBank (National Center for Biotechnology Information). To distinguish between different bacterial species and genera, we employed 97 to 100% sequence identity. The BLAST tool on the NCBI website (http://www.ncbi.nlm.nih.gov/) was used to analyze and identify each isolate's nucleotide sequences of the 16S rDNA gene. Using MEGA software version 11.0, the alignments were examined to create a phylogenetic tree and assess relationships between the sequences using the neighbor-joining approach (Tamura *et al*., 2013; Saitou & Nei, 1987).

2.6. Antagonistic Action in Vitro Screening

2.6.1. *Dual culture method*

In a PDA medium using a dual-culture approach, bacterial isolate was evaluated for in vitro biocontrol efficacy toward *R. solani*, *T. rubrum*, and *F. solani*. On one side of the PDA medium-filled plate (9 cm in diameter), a mycelial disc (5 mm in diameter) from a culture that had been growing for seven days was put. Thereafter, an endophytic bacteria isolate was streaked on the plate's other side. As a control, plates were infected only with the pathogen. The plates were

incubated for 7 days at 25 ± 2 °C with 12 hr of light and 12 hr of darkness. Fungal colonies' diameters were measured (Moreira *et al*., 2014). Then, the formula provided by Trivedi (2008) was used to compute the percentage of growth inhibition of the tested fungi: growth inhibition (%) = (R1 − R2/R1)×100, where R1 represents the radial growth of the control and R2 represents the radial growth of the fungus in dual culture.

2.6.2. *Effect of endophytic bacterium on fungal biomass*

The approach provided by Kim (2005) was modified to investigate the impact of endophytic bacteria on the biomass of fungus. The chosen endophytic bacteria were grown in NB media and incubated for 72 hours at 26 ± 2 °C on a rotary shaker. After that, it was filtered through a 0.22 m Millipore filter and centrifuged at 10,000 rpm for 15 minutes. 10% (v/v) of the strain's cell-free culture filtrates were added to the potato dextrose broth (PDB) medium in an Erlenmeyer flask (100 mL). One 5-mm-diameter plug of a mycelial disk from a 7-day-old culture of fungi was used to inoculate the Erlenmeyer flask, which was then incubated at 25 ± 2 °C for 14 days while being shaken at 150 rpm.

Cultures of the fungus grown without bacteria culture filtrates were used as the control. By the preweighed filter paper, 48-hour-grown dual cultures of the fungus and bacteria or the control culture (without bacteria) were passed to determine the differences in the dry weights between the two (Whatman No. 1). The filter sheets were weighed after drying for 24 hours at 70 °C. Using the formula (w1-w2/w1) 100, the weight loss of the test fungus was estimated as a percentage. where w1 (control value) is the weight of the test fungus in flasks devoid of bacteria (i.e., control flasks) and w2 is the weight of the fungus in sets that were contaminated with bacteria.

3. RESULTS

3.1. Isolation and Antagonistic Activity

The isolation of bacterial endophytes was carried out using fresh ***A. ×prunifolia*** roots. Surface sterilization was an essential step in eliminating epiphytic microorganisms from sample explants, and it worked well in our study because there was no growth on the control plate. Based on morphological traits, 25 different endophytic bacterial strains were isolated from the healthy roots and purified before being kept at 4 °C (Figure 1).

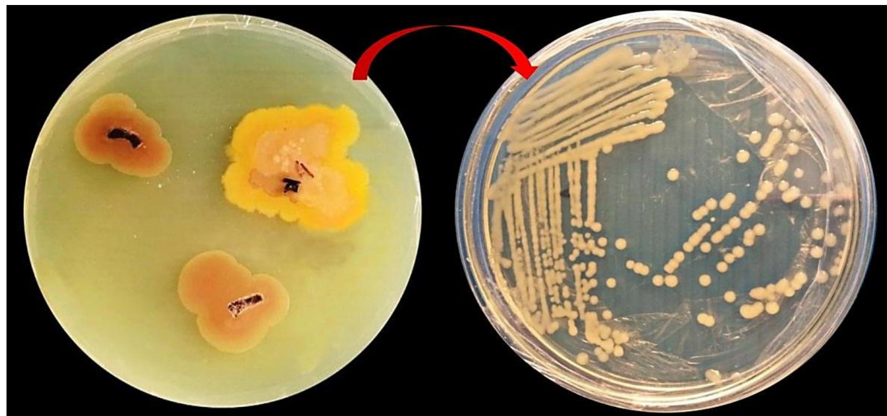

Figure 1. Growth of endophytic bacteria from pieces of *A. ×prunifolia* root on NA medium.

The isolates were evaluated their potential for serving as effective biological control agents using the dual-culture and Cell Free Supernatant (CFS) approach; these isolates were tested for their antagonistic activity against *F. solani*, *T. rubrum*, and *R. solani*. Among the isolates, strain LB2 demonstrated the most significant antagonistic activity against the target pathogens. The inhibition rates were 68.21±0.84, 39.35±0.59, and 48.35±0.28%, respectively (Table 1, Figure 2).

Table 1. After 7 days, LB2 strain in dual culture (live cells) inhibited the development of fungi's mycelia in in vitro testing.

Pathogenic fungi	Zone of inhibition (%)
Fusarium solani	68.21±0.84
Trichophyton rubrum	39.35±0.59
Rhizoctonia solani	48.35±0.28

Note: The assay was performed in triplicate and results are the mean of three values ± Standard Deviation.

Likewise, CFS treatment has also been shown to reduce the weight of tested pathogenic fungi. *F. solani*, *T. rubrum* and *R. solani* all exhibited substantial reductions in mycelial mass, with values of 34.53±0.22%, 30.18±0.73%, and 26.15±0.91%, respectively (Table 2). This result suggests that strain LB2 holds promising potential as a valuable biological control agent in managing the target pathogens.

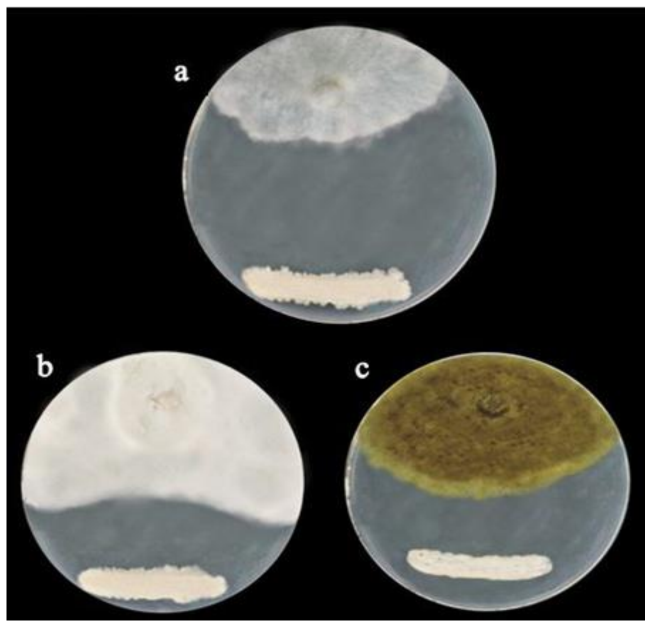

Figure 2. In vitro antagonistic activities of endophytic LB2 isolated from *A.* ×*prunifolia* roots against pathogenic fungi (a) *F. solani*, (b) against *T. rubrum*, (c) Antagonist activity against *R. solani*.

Table 2. After being incubated for 7 days at room temperature, LB2 (Cell-free culture filtrates) reduced the percentage of biomass of pathogenic fungus in PDB broth.

Pathogenic fungi	Fungal mycelia mass reduction (%)
Fusarium solani	34.53±0.22
Trichophyton rubrum	30.18±0.73
Rhizoctonia solani	26.15±0.91

Note: The assay was performed in triplicate and results are the mean of three values ± Standard Deviation.

3.2. Biochemical Characterization

The biochemical characteristics of the isolated bacterium play a crucial role in understanding its taxonomy, metabolic profile and potential applications. The biochemical analysis results are summarized in Table 3.

It was carried out based on the various tests. The bacterium LB2 exhibits several distinctive characteristics as revealed by Gram testing and various biochemical assays. It was Gram-negative, forming well-defined and even colonies on agar plates. LB2 lacks spore formation, suggesting alternative survival mechanisms. It ferments lactose and grows in the presence of bile salts and crystal violet, distinguishing it from non-lactose-fermenting Gram-negative

bacteria. It does not possess hemolytic factors, indicating non-pathogenic and non-hemolytic attributes. LB2 lacks urease, which is relevant in medical and environmental contexts. It can break down starch, potentially aiding in nutrient utilization and biotechnological applications. The presence of cytochrome c oxidase suggests it can use oxygen in aerobic respiration. It has catalase, which is important for handling reactive oxygen species. LB2 can break down gelatin, potentially relevant in nutrient cycling and bioremediation. The presence of tryptophanase is significant for identification. Fluorescence requires further investigation for source and significance. LB2 can metabolize specific organic compounds like Tween 40 and α-keto-butyric acid, exhibiting metabolic versatility. It can ferment various sugars and acetoacetic acid, enhancing its metabolic diversity for various applications. A few biochemical and physiological test findings are displayed in Figure 3.

Table 3. The isolate's biochemical and physiological testing.

Test	Resut
Gram staining	- / rod-shaped
Colony texture	Smooth
Spore	-
Growth on MacConkey	+
Blood Hemolysis	-
Urea hydrolysis	-
Starch hydrolysis	+
Oxidase	+
Catalase	+
Motility	+
Gelatin hydrolysis	+
Indole	+
Fluorescence	+
Tween 40	+
α-keto-butyric acid	+
Glucose	+
Lactose	+
Mannitol	+
Maltose	+
Acetoacetic acid	+

3.3. Identification of Bacterial Endophyte

The selected bacteria were identified by analyzing their 16S rRNA gene sequences. These associated bacteria displayed a complete match, showing 100% homology, with the gene sequences of *P. brassicacearum* available in the NCBI GenBank database. To further explore the evolutionary relationships among these bacteria, the top-scoring sequences were retrieved from the database and aligned with the 16S rRNA sequences of the endophytes to construct a phylogenetic tree.

This analysis was performed using Mega XI software, see Figure 4, and the resulting phylogram clearly distinguished between gram-positive and gram-negative endophytic bacteria. Specifically, all *P. brassicacearum* strains were found to cluster together as a distinct group (Cluster Ia) with a high bootstrap value of 100%. Notably, *Pseudomonas putida* was positioned as the outgroup in the constructed phylogenetic tree. This analysis sheds light on the evolutionary relationships and genetic diversity among the endophytic bacteria associated with *Aronia* roots.

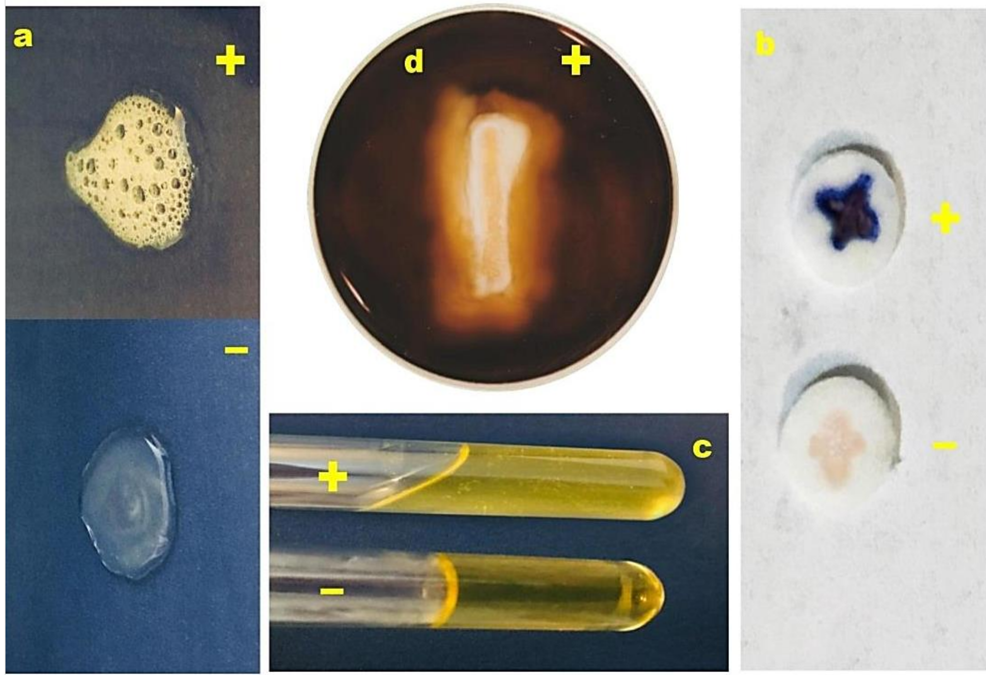

Figure 3. Biochemical and physiological tests a- Catalase test b- Oxidase test c- Gelatin hydrolysis test d- Starch hydrolysis on starch agar.

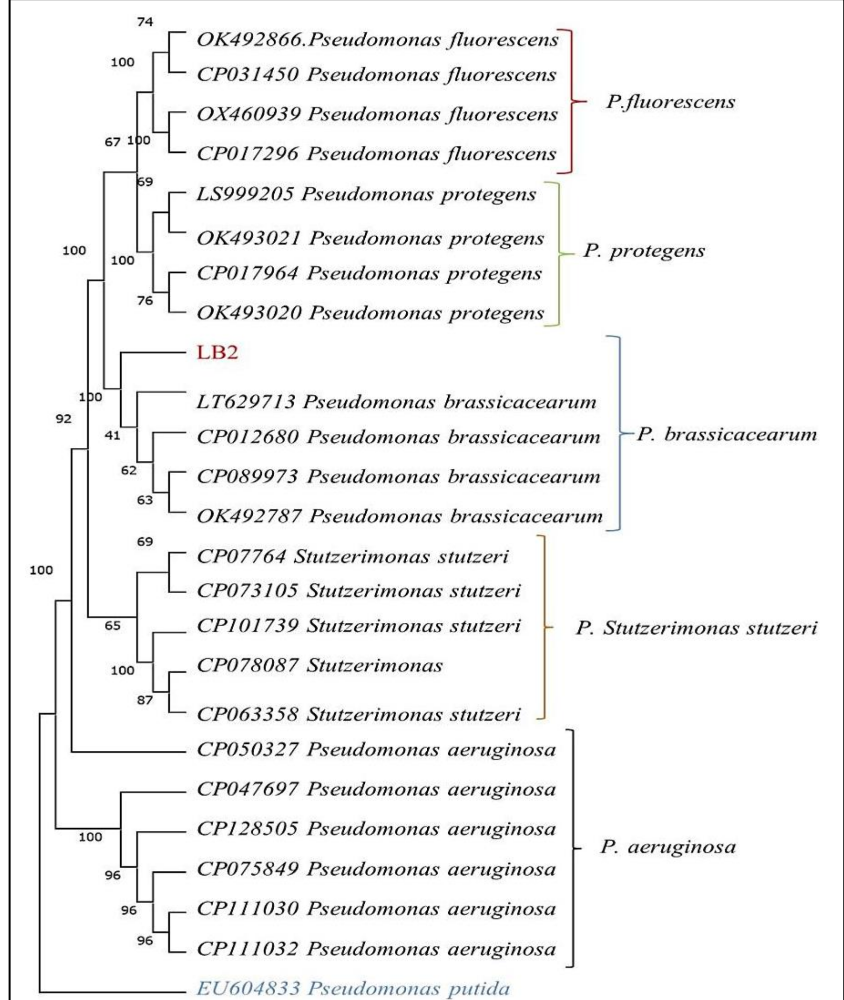

Figure 4. Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences illustrating connections between isolate LB2 (in red hue) and many other *Pseudomonas* sp. 1000 bootstrap replicates were used to create the tree in MEGA 11.0 using the neighbor-method.

4. DISCUSSION and CONCLUSION

A. prunifolia, also known as purple chokeberry, is a type of berry that is rich in phenolic compounds and antioxidants (Szopa *et al*., 2017; Kim *et al*., 2021). *Aronia* has been cultivated in Turkey, and as its output rises, the *Aronia* market is thought to be expanding (Akdemir *et al*., 2023). According to studies by Szopa *et al*. (2018), the *Aronia* plant contains a variety of medicinally significant compounds, such as chlorogenic acid, rosmarinic acid, and neochlorogenic acid that developed in the shoot and callus cultures of *A. prunifolia*.

Endophytic bacteria living in plants are important for maintaining plant health because they produce a variety of advantageous metabolites (Köberl *et al*., 2013; Musa *et al*., 2020). These molecules are excellent sources of biological activity. As previously indicated, biologically active chemicals produced in *A. ×prunifolia* significantly impact endophytic microorganisms that live inside plant tissue and their physiological processes (Köberl *et al*., 2013). So, these compounds can affect many biological processes.

In our study, strain LB2, isolated from the roots of *A. prunifolia*, was selected because it gave the highest inhibitory ability compared to the rest of the isolated strains. It was later characterized as *P. brassicacearum*. Many earlier investigations (Achouak *et al*., 2000; Ross *et al*., 2000; Chung *et al*., 2008; Bahmani *et al*., 2021) have already identified *P. brassicacearum* from other plants except *A. ×prunifolia* as endophytic bacteria. According to recent research, endophytic bacteria are an efficient biocontrol agent for various plant and human infections. Pseudomonas strains that have been isolated from various plant samples have demonstrated excellent effectiveness as plant-growth-promoting and biocontrol agents as well as makers of antimicrobial substances, antibiotics, enzymes, and volatile chemicals (Weller, 2007; Khan *et al*., 2022; Mustafa *et al*., 2024). In both greenhouse and field experiments, *P. brassicacearum* strain has been shown to be an effective biocontrol agent for preventing illness brought on by the plant-pathogenic fungus *Sclerotinia sclerotiorum* (Bhaskar *et al*., 2005; Berry *et al*., 2010). This bacterium generates a wide range of extracellular metabolites, such as oxidizing enzymes, hydrogen cyanide (HCN), and a brand-new lipopeptide known as sclerosin (Berry *et al*., 2010; Crawford *et al*., 2011). *Gaeumannomyces graminis* var. *tritici*, also known as the take-all fungus, is a destructive root disease that affects wheat; numerous investigations have demonstrated that *P. brassicacearum* isolates may stop this fungus from growing and may be useful as a biological control agent for plant diseases (Ross *et al*., 2000; Fromin *et al*., 2001).

In our study, diffusible metabolites and cell-free culture filtrates of *P. brassicacearum* strains have shown high inhibitory action against *R. solani*, *T. rubrum*, and *F. solani*. Many authors have shown that *P. brassicacearum* can stop the growth of fungal pathogens (Chung *et al*., 2008; Laveilhé *et al*., 2022). Studies on non-pathogenic microorganisms with antagonistic potentials have stimulated the search for an environmentally acceptable method of disease control. (Caulier *et al*., 2018). *Pseudomonas* spp. are significant organisms since they aggressively colonize different crops and exhibit a wide range of antagonistic activity against soil- and seed-borne diseases (Wang *et al*., 2018).

One of the most important assays for in vitro first antagonistic compound screening is the dual culture assay (Islam *et al*., 2018). A bacterial strain's antagonistic effects are frequently demonstrated by the development of inhibition zones between bacterial and fungal colonies (Ji *et al*., 2014) or by measuring the percentage of control mycelial growth that is inhibited by the bacterial colonies (Lee *et al*., 2017). According to an earlier study, Pseudomonas species compete for resources and habitats, synthesizing siderophores, secreting lytic enzymes, and inducing systemic resistance to plant diseases (Kang *et al*., 2015). On the other hand, broth-based treatments in vitro dual culture may be a superior way to assess the antagonistic potential of the bioagents because the broth media are favorable environments from all potentially interacting locations for the antagonisms (Trivedi *et al*., 2008). Many different gram-negative bacteria have been used in the treatment of fungal infections. These bacteria function as antifungal agents by synthesizing siderophores, salicylic acid, antibiotics, and volatile

byproducts such as hydrogen cyanide (Manwar *et al*., 2004; Afsharmanesh *et al*., 2006; Correa *et al*., 2022; Shahid *et al*., 2022).

In this study, it was determined that the isolate *P. brassicacearum* LB2 reduced the biomass of all studied fungal organisms by producing diffusible chemicals in broth media. However, the extracellular filtrates obtained from *P. brassicacearum* have presented low antifungal activity compared to dual culture assay. The Gram-negative bacteria isolated in this study likely generate antifungal volatile chemicals or enzymatic activity that are lost during the extraction of the filtrates. Therefore, it may be inferred that the compounds that cause the inhibition must exist in the presence of bacteria and may be linked to the live bacterial properties. The findings reported here suggest that *P. brassicacearum* may be transformed into a biocontrol-friendly product. In fact, endophytic fungi within the host plants are a flexible reservoir of numerous bioactive metabolites and may be used in contemporary agriculture, industry and medicine.

The findings of this study highlight the significance of endophytic bacteria, particularly *P. brassicacearum*, isolated from *A. ×prunifolia* roots as natural antifungal allies in the ongoing struggle against plant fungal infections. Their multifaceted mechanisms of action, including resource competition, antibiosis, induced systemic resistance, and mycoparasitism, offer a sustainable and environmentally responsible approach to protecting plants. Due to the search for alternatives to chemical fungicides, this research will reinforce the importance of eco-friendly alternatives in plant protection and medical practices.

This study may also pave the way to benefit from this endophyte in different areas. So, this contribution highlights their potential as potent biocontrol agents and opens new avenues for harnessing their capabilities in agricultural and horticultural practices.

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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

Luay B. Mustafa: Design, Experimental Studies, Writing the Original Draft, and Manuscript Review. **Ahmed İ. N. Al-Bayati**: Analysis, Manuscript Checking, and Correction. **Dunya Albayati**: Literature Survey, Data Collection, and Data Editing. **İbrahim Özkoç**: Supervision, Validation, and Editing.

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REFERENCES

Achouak, W., Sutra, L., Heulin, T., Meyer, J.M., Fromin, N., Degraeve, S., ... & Gardan, L. (2000). *Pseudomonas brassicacearum* sp. nov. and *Pseudomonas thivervalensis* sp. nov., two root-associated bacteria isolated from Brassica napus and *Arabidopsis thaliana*. *International Journal of Systematic and Evolutionary Microbiology*, *50*(1), 9-18. https://doi.org/10.1099/00207713-50-1-9

Adeleke, B.S., & Babalola, O.O. (2022). Meta-omics of endophytic microbes in agricultural biotechnology. *Biocatalysis and Agricultural Biotechnology*, *42*, 102332. https://doi.org/10.1016/j.bcab.2022.102332

Afsharmanesh, H., Ahmadzadeh, M., & Sharifi-Tehrani, A. (2006). Biocontrol of Rhizoctonia solani, the causal agent of bean damping-off by fluorescent pseudomonads. *Communications in Agricultural and Applied Biological Sciences*, *71*(3 Pt B), 1021-1029.

Akdemir, S., Torçuk, A.I., & Uysal Seçkin, G. (2023). Determination of Quality Parameters of *Aronia Melanocarpa* During Cold Storage. Erwerbs-Obstbau, 1-7. https://doi.org/10.1007/s10341-023-00845-4

Alsohiby, F.A.A., Yahya, S., & Humaid, A.A. (2016). Screening of soil isolates of bacteria for antagonistic activity against plant pathogenic fungi. *PSM Microbiology, 1*(1), 5-9.

Anand, U., Pal, T., Yadav, N., Singh, V.K., Tripathi, V., Choudhary, K.K., ... & Singh, A.K. (2023). Current scenario and future prospects of endophytic microbes: promising candidates for abiotic and biotic stress management for agricultural and environmental sustainability. *Microbial Ecology*, 1-32. https://doi.org/10.1007/s00248-023-02190-1

Bahmani, K., Hasanzadeh, N., Harighi, B., & Marefat, A. (2021). Isolation and identification of endophytic bacteria from potato tissues and their effects as biological control agents against bacterial wilt. *Physiological and Molecular Plant Pathology*, *116*, 101692. https://doi.org/10.1016/j.pmpp.2021.101692

Berg, G., & Hallmann, J. (2006). Control of plant pathogenic fungi with bacterial endophytes. *Microbial Root Endophytes*, 53-69. https://doi.org/10.1007/3-540-33526-9

Berg, G., Krechel, A., Ditz, M., Sikora, R.A., Ulrich, A., & Hallmann, J. (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiology Ecology*, *51*(2), 215-229. https://doi.org/10.1016/j.femsec.2004.08.006

Berry, C., Fernando, W.D., Loewen, P.C., & De Kievit, T.R. (2010). Lipopeptides are essential for Pseudomonas sp. DF41 biocontrol of *Sclerotinia sclerotiorum*. *Biological Control*, *55*(3), 211-218. https://doi.org/10.1016/j.biocontrol.2010.09.011

Bhaskar, P.V., Grossart, H.P., Bhosle, N.B., & Simon, M. (2005). Production of macroaggregates from dissolved exopolymeric substances (EPS) of bacterial and diatom origin. *FEMS Microbiology Ecology*, *53*(2), 255-264. https://doi.org/10.1016/j.femsec.2004.12.013

Boonman, N., Chutrtong, J., Wanna, C., Boonsilp, S., & Chunchob, S. (2023). Antimicrobial activities of endophytic bacteria isolated from *Ageratum conyzoides* Linn. *Biodiversitas Journal of Biological Diversity*, *24*(4). https://doi.org/10.13057/biodiv/d240405

Cao, L., Qiu, Z., You, J., Tan, H., & Zhou, S. (2005). Isolation and characterization of endophytic streptomycete antagonists of *Fusarium* wilt pathogen from surface-sterilized banana roots. *FEMS Microbiology Letters*, *247*(2), 147-152. https://doi.org/10.1016/j.femsle.2005.05.006

Caulier, S., Gillis, A., Colau, G., Licciardi, F., Liépin, M., Desoignies, N., ..., & Bragard, C. (2018). Versatile antagonistic activities of soil-borne Bacillus spp. and *Pseudomonas* spp. against *Phytophthora infestans* and other potato pathogens. *Frontiers in Microbiology*, *9*, 143. https://doi.org/10.3389/fmicb.2018.00143

Celka, Z., & Szkudlarz, P. (2010). Spontaneous occurrence and dispersion of *Aronia* x *prunifolia* [Marshall] rehder [Rosaceae] in Poland on the example of the'Bagna'bog complex near Chlebowo [western Poland]. *Acta Societatis Botanicorum Poloniae*, *79*(1), 37-42.

Chen, C., Bauske, E.M., Musson, G., Rodriguezkabana, R., & Kloepper, J.W. (1995). Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. *Biological Control*, *5*(1), 83-91. https://doi.org/10.1006/bcon.1995.1009

Cho, S.J., Park, S.R., Kim, M.K., Lim, W.J., Ryu, S.K., An, C.L., ..., & Yun, H.D. (2002). Endophytic Bacillus sp. isolated from the interior of balloon flower root. *Bioscience, Biotechnology, and Biochemistry*, *66*(6), 1270-1275. https://doi.org/10.1271/bbb.66.1270

Chung, B.S., Aslam, Z., Kim, S.W., Kim, G.G., Kang, H.S., Ahn, J.W., & Chung, Y.R. (2008). A bacterial endophyte, *Pseudomonas brassicacearum* YC5480, isolated from the root of

Artemisia sp. producing antifungal and phytotoxic compounds. *The Plant Pathology Journal*, *24*(4), 461-468. https://doi.org/10.5423/PPJ.2008.24.4.461

Cipriano, M.A.P., Freitas-Iório, R.D.P., Dimitrov, M.R., de Andrade, S.A.L., Kuramae, E.E., & Silveira, A.P.D.D. (2021). Plant-growth endophytic bacteria improve nutrient use efficiency and modulate foliar N-metabolites in sugarcane seedling. *Microorganisms*, *9*(3), 479. https://doi.org/10.3390/microorganisms9030479

Correa, P.A., Nosheen, A., Yasmin, H., & Ansari, M.J. (2022). Antifungal Antibiotics Biosynthesized by Major PGPR. In *Secondary Metabolites and Volatiles of PGPR in Plant-Growth Promotion* (pp. 199-247). Cham: Springer International Publishing. https://doi.org/10.36721/PJPS.2023.36.3.SP.927-934.1

Crawford, J.M., Portmann, C., Kontnik, R., Walsh, C.T., & Clardy, J. (2011). NRPS substrate promiscuity diversifies the xenematides. *Organic Letters*, *13*(19), 5144-5147. https://doi.org/10.1021/ol2020237

Dalton, D.A., Kramer, S., Azios, N., Fusaro, S., Cahill, E., & Kennedy, C. (2004). Endophytic nitrogen fixation in dune grasses (*Ammophila arenaria* and *Elymus mollis*) from Oregon. *FEMS microbiology ecology*, *49*(3), 469-479. https://doi.org/10.1016/j.femsec.2004.04.010

Fromin, N., Achouak, W., Thiéry, J.M., & Heulin, T. (2001). The genotypic diversity of *Pseudomonas brassicacearum* populations isolated from roots of *Arabidopsis thaliana*: influence of plant genotype. *FEMS Microbiology Ecology*, *37*(1), 21-29. https://doi.org/10.1111/j.1574-6941.2001.tb00849.x

Granér, G., Persson, P., Meijer, J., & Alström, S. (2003). A study on microbial diversity in different cultivars of *Brassica napus* in relation to its wilt pathogen, *Verticillium longisporum*. *FEMS Microbiology Letters*, *224*(2), 269-276. https://doi.org/10.1016/S0378-1097(03)00449-X

Hall, T.A. (1999). Bio Edit; a user-friendly biological sequence aliment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symp. Ser.* (Vol. 41, p. 95).

Heuer, H., Krsek, M., Baker, P., Smalla, K., & Wellington, E. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology*, *63*(8), 3233-3241. https://doi.org/10.1128/aem.63.8.3233-3241.1997

Ho, G.T., Bräunlich, M., Austarheim, I., Wangensteen, H., Malterud, K.E., Slimestad, R., & Barsett, H. (2014). Immunomodulating activity of *Aronia melanocarpa* polyphenols. *International Journal of Molecular Sciences*, *15*(7), 11626-11636. https://doi.org/10.3390/ijms150711626

Islam, M.A., Nain, Z., Alam, M.K., Banu, N.A., & Islam, M.R. (2018). In vitro study of biocontrol potential of rhizospheric Pseudomonas aeruginosa against *Fusarium oxysporum* f. sp. *cucumerinum*. *Egyptian journal of Biological Pest control*, *28*, 1-11. https://doi.org/10.1186/s41938-018-0097-1

Ji, S.H., Gururani, M.A., & Chun, S.C. (2014). Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. *Microbiological research*, *169*(1), 83-98. https://doi.org/10.1016/j.micres.2013.06.003

Kang, S.M., Radhakrishnan, R., & Lee, I.J. (2015). *Bacillus amyloliquefaciens* subsp. *plantarum* GR53, a potent biocontrol agent resists Rhizoctonia disease on Chinese cabbage through hormonal and antioxidants regulation. *World Journal of Microbiology and Biotechnology*, *31*, 1517-1527. https://doi.org/10.1007/s11274-015-1896-0

Khan, M.S., Gao, J., Zhang, M., Xue, J., & Zhang, X. (2022). Pseudomonas aeruginosa Ld-08 isolated from *Lilium davidii* exhibits antifungal and growth-promoting properties. Plos one, 17(6), e0269640. https://doi.org/10.1371/journal.pone.0269640

Kim, D.W., Han, H.A., Kim, J.K., Kim, D.H., & Kim, M.K. (2021). comparison of phytochemicals and antioxidant activities of berries cultivated in Korea: Identification of phenolic compounds in *Aronia* by HPLC/Q-TOF MS. *Preventive Nutrition and Food Science*, *26*(4), 459. https://doi.org/10.3746%2Fpnf.2021.26.4.459

Kim, J.D. (2005). Antifungal activity of lactic acid bacteria isolated from Kimchi against *Aspergillus fumigatus*. *Mycobiology*, *33*(4), 210-214. https://doi.org/10.4489/MYCO.2005.33.4.210

Köberl, M., Ramadan, E.M., Adam, M., Cardinale, M., Hallmann, J., Heuer, H., ... & Berg, G. (2013). *Bacillus* and *Streptomyces* were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt. *FEMS Microbiology Letters*, *342*(2), 168-178. https://doi.org/10.1111/1574-6968.12089

Kulling, S.E., & Rawel, H.M. (2008). Chokeberry (*Aronia melanocarpa*)–A review on the characteristic components and potential health effects. *Planta Medica*, *74*(13), 1625-1634. https://doi.org/10.1055/s-0028-1088306

Laveilhé, A., Fochesato, S., Lalaouna, D., Heulin, T., & Achouak, W. (2022). Phytobeneficial traits of rhizobacteria under the control of multiple molecular dialogues. *Microbial Biotechnology*, *15*(7), 2083-2096. https://doi.org/10.1111/1751-7915.14023

Lee, T., Park, D., Kim, K., Lim, S.M., Yu, N.H., Kim, S., ..., & Kim, J.C. (2017). Characterization of Bacillus amyloliquefaciens DA12 showing potent antifungal activity against mycotoxigenic *Fusarium* species. *The Plant Pathology Journal*, *33*(5), 499. https://doi.org/10.5423%2FPPJ.FT.06.2017.0126

Mahaffee, W.F., & Kloepper, J.W. (1997). Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.). *Microbial Ecology*, *34*, 210-223. https://doi.org/10.1007/s002489900050

Manwar, A.V., Khandelwal, S.R., Chaudhari, B.L., Meyer, J.M., & Chincholkar, S.B. (2004). Siderophore production by a marine *Pseudomonas aeruginosa* and its antagonistic action against phytopathogenic fungi. *Applied Biochemistry and Biotechnology*, *118*, 243-251. https://doi.org/10.1385/ABAB:118:1-3:243

Miyamoto, T., Kawahara, M., & Minamisawa, K. (2004). Novel endophytic nitrogen-fixing clostridia from the grass *Miscanthus sinensis* as revealed by terminal restriction fragment length polymorphism analysis. *Applied and Environmental Microbiology*, *70*(11), 6580-6586. https://doi.org/10.1128/AEM.70.11.6580-6586.2004

Moreira, R.R., Nesi, C.N., & De Mio, L.L.M. (2014). *Bacillus* spp. and *Pseudomonas putida* as inhibitors of the *Colletotrichum acutatum* group and potential to control *Glomerella* leaf spot. *Biological control*, *72*, 30-37. https://doi.org/10.1016/j.biocontrol.2014.02.001

Musa, Z., Ma, J., Egamberdieva, D., Abdelshafy Mohamad, O.A., Abaydulla, G., Liu, Y., & Li, L. (2020). Diversity and antimicrobial potential of cultivable endophytic actinobacteria associated with the medicinal plant *Thymus roseus*. *Frontiers in Microbiology*, *11*, 191. https://doi.org/10.3389/fmicb.2020.00191

Mustafa, L.B., Al-Bayati, A.I.N., & Özkoç, I. (2024). Salt-tolerant endophytic Pseudomonas putida isolated from *Aronia prunifolia* root with plant growth-promoting potential. *World News of Natural Sciences*, *53*, 212-222.

Naranjo, H.D., Rat, A., De Zutter, N., De Ridder, E., Lebbe, L., Audenaert, K., & Willems, A. (2023). Uncovering Genomic Features and Biosynthetic Gene Clusters in Endophytic Bacteria from Roots of the Medicinal Plant *Alkanna tinctoria* Tausch as a Strategy to Identify Novel Biocontrol Bacteria. *Microbiology Spectrum*, *11*(4), e00747-23. https://doi.org/10.1128/spectrum.00747-23

Nielsen, T.H., Sørensen, D., Tobiasen, C., Andersen, J. B., Christophersen, C., Givskov, M., & Sørensen, J. (2002). Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. *Applied and environmental microbiology*, *68*(7), 3416-3423. https://doi.org/10.1128/AEM.68.7.3416-3423.2002

Pascual, P. (2000). Characterization of *Rhizoctonia solani* isolates causing banded leaf and sheath blight in corn by conventional and PCR-based techniques. *Plant Pathology*, *49*, 108-118.

Rana, K.L., Kour, D., Kaur, T., Devi, R., Yadav, A., & Yadav, A.N. (2021). Bioprospecting of endophytic bacteria from the Indian Himalayas and their role in plant growth promotion of maize (Zea mays L.). *Journal of Applied Biology and Biotechnology*, *9*(3), 41-50. http://dx.doi.org/10.7324/JABB.2021.9306

Ross, I.L., Alami, Y., Harvey, P.R., Achouak, W., & Ryder, M.H. (2000). Genetic diversity and biological control activity of novel species of closely related pseudomonads isolated from wheat field soils in South Australia. *Applied and Environmental Microbiology*, *66*(4), 1609-1616. https://doi.org/10.1128/AEM.66.4.1609-1616.2000

Safaa, A.L., & Qaysi, Z.A.T. (2016). Levan production using Pseudomonas brassicacearum isolated from rhizosphere soil of cowpea farm in Iraq. *Iraqi journal of biotechnology*, *15*(1).

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Salazar, O., Schneider, J.H., Julian, M.C., Keijer, J., & Rubio, V. (1999). Phylogenetic subgrouping of Rhizoctonia solani AG 2 isolates based on ribosomal ITS sequences. *Mycologia*, *91*(3), 459-467. https://doi.org/10.1080/00275514.1999.12061039

Santra, H.K., & Banerjee, D. (2023). Antifungal activity of volatile and non-volatile metabolites of endophytes of Chloranthus elatior Sw. *Frontiers in Plant Science*, *14*, 1156323. https://doi.org/10.3389/fpls.2023.1156323

Shahid, M., Singh, U.B., Ilyas, T., Malviya, D., Vishwakarma, S.K., Shafi, Z., ... & Singh, H.V. (2022). Bacterial inoculants for control of fungal diseases in *Solanum lycopersicum* L.(tomatoes): a comprehensive overview. *Rhizosphere Microbes: Biotic Stress Management*, 311-339. https://doi.org/10.1007/978-981-19-5872-4_15

Siciliano, S.D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., ... & Greer, C.W. (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Applied and Environmental Microbiology*, *67*(6), 2469-2475. https://doi.org/10.1128/AEM.67.6.2469-2475.2001

Singh, P., Singh, R.K., Guo, D.J., Sharma, A., Singh, R.N., Li, D.P., ..., & Li, Y.R. (2021). Whole genome analysis of sugarcane root-associated endophyte *Pseudomonas aeruginosa* B18 - A plant growth-promoting bacterium with antagonistic potential against *Sporisorium scitamineum*. *Frontiers in Microbiology*, *12*, 628376. https://doi.org/10.3389/fmicb.2021.628376

Sneath, P.H.A. (1992). Preface to the present edition. In *International Code of Nomenclature of Bacteria: Bacteriological Code, 1990 Revision*. ASM Press.

Sturz, A.V. (1999). Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. *Plant Pathology*, *48*, 360-369. https://doi.org/10.1046/j.1365-3059.1999.00351.x

Szopa, A., Kokotkiewicz, A., Kubica, P., Banaszczak, P., Wojtanowska-Krośniak, A., Krośniak, M., ..., & Ekiert, H. (2017). Comparative analysis of different groups of phenolic compounds in fruit and leaf extracts of *Aronia* sp.: *A. melanocarpa, A. arbutifolia*, and *A.*× *prunifolia* and their antioxidant activities. *European Food Research and Technology*, *243*, 1645-1657. https://doi.org/10.1007/s00217-017-2872-8

Szopa, A., Kubica, P., Snoch, A., & Ekiert, H. (2018). High production of bioactive depsides in shoot and callus cultures of Aronia arbutifolia and Aronia× prunifolia. *Acta Physiologiae Plantarum*, *40*, 1-11. https://doi.org/10.1007/s11738-018-2623-x

Taheri, R., Connolly, B.A., Brand, M.H., & Bolling, B.W. (2013). Underutilized chokeberry (*Aronia melanocarpa, Aronia arbutifolia, Aronia prunifolia*) accessions are rich sources of anthocyanins, flavonoids, hydroxycinnamic acids, and proanthocyanidins. *Journal of agricultural and food chemistry*, *61*(36), 8581-8588. https://doi.org/10.1021/jf402449q

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729. https://doi.org/10.1093/molbev/mst197

Trivedi, P., Pandey, A., & Palni, L.M.S. (2008). In vitro evaluation of antagonistic properties of *Pseudomonas corrugata*. *Microbiological Research*, *163*(3), 329-336. https://doi.org/10.1016/j.micres.2006.06.007

Wang, X., Wang, C., Li, Q., Zhang, J., Ji, C., Sui, J., ..., & Liu, X. (2018). Isolation and characterization of antagonistic bacteria with the potential for biocontrol of soil-borne wheat diseases. *Journal of Applied Microbiology*, *125*(6), 1868-1880. https://doi.org/10.1155/2019/3638926

Weller, D.M. (2007). Pseudomonas biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology, 97(2), 250-256. https://doi.org/10.1094/PHYTO-97-2-0250

Yang, K., Qin, Q., Liu, Y., Zhang, L., Liang, L., Lan, H., ..., & Wang, S. (2016). Adenylate cyclase AcyA regulates development, aflatoxin biosynthesis and fungal virulence in *Aspergillus flavus*. *Frontiers in Cellular and Infection Microbiology*, *6*, 190. https://doi.org/10.3389/fcimb.2016.00190

Zinniel, D.K., Lambrecht, P., Harris, N.B., Feng, Z., Kuczmarski, D., Higley, P., ... & Vidaver, A.K. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and environmental microbiology*, *68*(5), 2198-2208. https://doi.org/10.1128/AEM.68.5.2198-2208.2002

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journal homepage: https://dergipark.org.tr/en/pub/ijsm **Research Article**

Assessment of the differences of hematological variables and their correlation with glycemic control among type 2 diabetes mellitus patients in Iraq: Comparative cross-sectional study

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Abstract: This research aimed to assess the hematological traits of male adults diagnosed with diabetes and investigate the relationship between blood sugar levels with hematological factors among patients. A cross-sectional comparison study was conducted at Fallujah Teaching Hospital from April 1 to July 30, 2023. The research comprised 185 volunteers, including 125 individuals with type 2 diabetes mellitus (65 with well blood sugar levels and 60 with poorly-regulated blood sugar levels) and 60 healthy individuals serving as controls. The evaluation of hematological parameters was conducted using Swelab-Alfa. An independent T-test was used for assessment. The patients exhibited substantially decreased mean absolute lymphocyte count, Hct, MCHC, and PLT values compared to the control group. The diabetic group had significantly higher mean values for total neutrophil count, absolute basophil counts, RDWSD, RDWCV, PDW, PLCR, and MPV than the control group. Patients with poor glycemic control had substantially elevated levels of Mon, Eos, Bas, MCHC, PLT, MPV, PLCR, and PCT. In contrast, individuals with poor glycemic control had substantially lower levels of Neu, RBC count, and PDW. The findings demonstrated a statistically significant positive connection between neutrophil count, MCV, MCH, MCHC, PDW, MPV, PLCR, and PCT with FBG. Lym, RBC count, and Hct exhibited a statistically significant inverse connection with FBG in individuals with type 2 diabetes mellitus (T2DM). This research demonstrated a notable impact of diabetes mellitus, poor glycemic control, and fasting blood glucose levels on some hematological markers.

1. INTRODUCTION

Diabetes mellitus (DM) is a rapidly worsening worldwide medical issue and one of the top non-communicable illnesses that leaders around the globe are focusing on addressing, it has been placed ninth among diseases that cause mortality (World Health Organization, 2016). Diabetes, a metabolic disorder, has been identified as two cardinal kinds, 1 and 2 diabetes. Type 2 diabetes (T2DM), which constitutes a significant proportion of DM cases, accounting for 90-95% of

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them, is typified by the presence of peripheral insulin resistance or decreased insulin production (American Diabetes Association, 2014).

In 2021, there was a substantial rise in the number of persons diagnosed with diabetes mellitus (DM), reaching 537 million. Diabetes is more common in low- and middle-income countries, constituting over 75% of the global prevalence. Moreover, experts predict that the worldwide diabetes population will reach 783 million by 2045 (International Diabetes Federation, 2021). The prevalence of diabetes has been steadily rising across the countries of the Middle East and northern Africa. A recent global study indicates that the Middle East and North Africa region has a diabetes prevalence rate of 16.2%, impacting around 73 million individuals. In Iraq, the prevalence of diabetes among adults is particularly estimated to be about 9.4%, affecting roughly 2.0114 million persons (International Diabetes Federation, 2021). Diabetes mellitus is characterized by a multitude of pathological alterations, encompassing derangements in metabolism, cellular processes, and blood constituents that lead to vascular consequences (Agu, 2018).

The Hematological indices have been shown to alter in T2DM patients, including changes to platelet counts, platelet indices, white blood cell (WBC), and red blood cell (RBC) shape, function, and metabolism (Antwi-Baffour *et al.*, 2018). According to the study's findings, chronic hyperglycemia in diabetes lead to increase production of reactive oxygen species (ROS) and glycation processes which result changes in hematological parameters. Moreover, increased ROS generation and glycation processes in diabetes patients cause oxidative stress linked to tissue damage and hematological alterations, including erythrocyte dysregulation, Activated WBCs, boosted WBC count, and PLT hyperactivity, (Çakırca *et al.*, 2019; Shin *et al.*, 2020).

Inflammation and endothelial cell dysfunction have been indicated by some hematological parameters among diabetic patients such as white blood cell count, red blood cell distribution width (RDW), mean platelet volume (MPV), platelet distribution width, and platelet count (Asmah *et al.*, 2015; Kaur *et al.*, 2018). Besides, glucose level in T2DM might regulate by Monitoring patients tests may prevent diabetes complication (American Diabetes Association, 2014; Agu, 2018; Çakırca et al., 2019).

The alterations of hematological indices among patient with T2DM were clarified by various researchers in different regions, but the results were inconsistent. Some studies show no significant variation in RBC indices, WBC count, and platelet count between diabetic patients and healthy controls (Alhadas et al., 2016; Kizilgul et al., 2018; Osman & Mansour, 2013). However, other research has revealed that diabetic patients exhibit significantly higher RBC, WBC, and PLT indices than control subjects (Jabeen et al., 2013). Besides, some investigations have communicated that RBC indices, except RDW, are significantly lower in the diabetic group compared to the control group, while WBC and PLT indices are substantially higher in the diabetic group (Biadgo et al., 2016; Shehri, 2017). These conflicting results indicate that more research is needed to investigate hematological abnormalities in diabetic patients, and the effect of other variables such as age, gender, and glycemic control on blood parameters in diabetic patients should be considered. Also, The Differences in cultural differences and study design, physiological conditions, obesity, smoking, alcoholism and sample features could impact hematology markers such as hemoglobin levels, RBC, WBC, and Platelet indices. In addition, Hemoglobin (Hgb) values below 13.0 g/dL for men are considered anemia (Cappellini & Motta, 2015). Most investigations in previous studies do not exclude smokers, alcoholism and abnormal body mass index. Therefore, this study looks at hematological variables in adult males between diabetic and healthy, as well as comparing controlling and uncontrolled diabetes, and also the relationship between fasting glucose level and hematological variables among patients.

2. MATERIAL and METHODS

2.1. Study Population and Participants

The study was a cross-sectional investigation that was carried out from 1st April to 30th July of 2023.The research was carried out at the Fallujah Teaching Hospital, Fallujah, Iraq. The study consisted of a sample of 165 male subjects who had type 2 Diabetes, as well as a control group of 60 healthy males. The diabetic patients had been living with the disease between 5 and 10 years. All participants were male and the ages (40 – 50), with normal body mass index. The control group appeared healthy with no signs of diabetes and were not suffering from any other chronic diseases. Individuals who had any chronic ailments, such as liver diseases, smokers, alcoholics, abnormal body mass index, women, or prediabetics were exclusion criteria.

The patient group was segregated into two distinct groups based on their fasting blood glucose (FBS) as good glycemic control is comprised of individuals whose FBS levels were under 152 mg/dL and poor glycemic control had FBS levels exceeding 152 mg/dL (American Diabetes Association, 2019). The data collection protocol utilized in this study was based on a systematic questionnaire comprising a set of well-constructed inquiries. The questionnaire sought data on the participant's age, gender, name, diabetes status, and consumption of lipid-lowering drugs and haematinics

2.2. Sampling Procedure

Blood samples were acquired from study volunteers in both groups in a supervised and sterilization. In evaluating haematology indices, the first 3 millilitres (ml) from blood samples were immediately placed in a red test tube (containing K2EDTA), an anticoagulant. All blood samples were thoroughly checked for hemolysis and clots before analysis. The samples were then appropriately blended. After that, 50 microliters of each sample were examined in under an hour utilizing the Swelab-Alfa automated haematology analyzer for the instrumental analysis. We meticulously documented and archived these data for future statistical studies.

The glucose level was estimated via chemical analysis by applying a small amount of blood onto the ACCU-CHEK Glucose meter strip. The glucose level was determined using this procedure.

2.3. Data Analysis

Data was gathered through notebooks and subsequently transferred to a computer, which was kept secure and confidential. Statistical Package for Social Sciences (SPSS, Version 25) was used to compare the data between groups. The study conducted a statistical analysis to compare the haematological parameters of participants with and without diabetes. They used an independent sample t-test for this purpose. Similarly, the analysis compared diabetic patients with good glycemic control to those with poor glycemic control. The findings regarding the mean value ± standard deviation (*SD*) were reported. Pearson's correlation was utilized to determine the relationship between the obtained haematological indices and fasting glucose. Statistical significance was considered to be a p-value below 0.05.

3. RESULTS

3.1. Comparison of Hematology Parameters Between Groups

The current study employed an independent t-test to scrutinize any dissimilarity between the patients and control groups in the average of the haematological variables (Table 1). WBC, lymphocyte count, and basophil count were notably elevated in diabetic patients, while lymphocyte count was significantly low in the diabetes group. However, the difference was insignificant in eosinophil count and monocyte count. Interestingly, the patients' RBC, Hct, and MCHC significantly decreased. At the same time, the RDWSD and RDWCV were elevated considerably among the patients. Furthermore, the means of PDW, MPV, PLCR, and glucose

were markedly increased in the diabetes group, but the PLT counts were significantly lowered in the diabetic group.

Table 1. Comparison of hematology Variables between case study and controls at Fallujah hospital, Iraq, from 1st April to 30th July 2023 (n = 185).

Variables	Control n = (60)	Case n = (125)	*p*-value
Red blood cell indices			
RBC (10^6/µL) Mean ± *SD*	4.726 (0.81176)	4.485 (0.74144)	0.264
Hct (%) Mean ± *SD*	39.28 (3.12447)	37.3696 (5.22982)	0.045
Hgb (g/dL) Mean ± *SD*	13.07 (1.09642)	12.0184 (1.65653)	0.129
MCV (fL) Mean ± *SD*	83.78 (9.70837)	83.9488(6.62536)	0.143
MCH (Pg) Mean ± *SD*	27.93 (3.66052)	27.028 (2.37023)	0.064
MCHC (g/dL) Mean ± *SD*	33.33 (0.85337)	32.1744 (1.16196)	0.002
RDWSD (fL) Mean ± *SD*	39.63 (2.93248)	43.2984 (6.53715)	0.000
RDWCV (%) Mean ± *SD*	13.21 (1.88470)	14.2224 (2.10984)	0.025
White blood cell indices			
WBC (10^3/µL) Mean ± *SD*	7.5760 (1.86997)	8.8781 (3.04349)	0.001
Lym (10^3/µL) Mean ± *SD*	2.2020 (0.51891)	2.0742 (0.83454)	0.001
Neu (10^3/µL) Mean ± *SD*	4.2970 (1.85443)	5.9990 (3.75224)	0.000
Mon (10^3/µL) Mean ± *SD*	0.5570 (0.16230)	0.5925 (0.19423)	0.485
Eos (10^3/µL) Mean ± *SD*	0.2640 (0.13953)	0.2088 (0.17925)	0.590
Bas (10^3/µL) Mean ± *SD*	0.0890 (0.017211)	0.0252 (0.01484)	0.000
Platelet indices			
PLT (10^3/µL) Mean ± *SD*	294.1 (61.29360)	292.488 (83.07882)	0.027
PDW (fL) Mean ± *SD*	11.7 (2.13970)	12.684 (2.67679)	0.009
MPV (fL) Mean ± *SD*	10.206 (.91669)	10.468 (1.18632)	0.021
PLC-R (%)Mean ± *SD*	26.1 (7.36202)	28.7616 (9.43224)	0.005
PCT (%) Mean ± *SD*	0.2890 (0.06650)	0.2943 (0.08240)	0.923

Abbreviations: RBC; red blood cells, Hgb; hemoglobin, Hct; hematocrit, MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration, RDW- *SD*; red cell distribution width standard deviation, RDW-CV; red cell distribution width coefficient of variation, Lym; lymphocyte, Nue; neutrophils , Mon; monocytes, Eos; eosinophil, Bas; basophil , PDW; platelet distribution width, PLC-R; platelet large cell ratio, FBG; fasting blood glucose, *SD*; standard deviation

3.2. Comparison of Hematology Variables Between Groups In Patients

The research has shown notable variations in haematological parameters across groups (Table 2). The research shows that the average levels of Mon, Eos, Bas, MCHC, PLT, MPV, PLCR, PCT, and FBG were notably elevated in individuals with poor glycemic control. In contrast, the average values of Neu, RBC count, and PDW were lower in individuals with poor glycemic control. However, the two groups observed no statistically significant changes in other haematological parameters.

Table 2. Comparison of hematology Variables between good glycemic control and poor glycemic control in case study at Fallujah Hospital, Iraq, from 1st April to 30th July 2023 (n = 125).

Parameters	Glycemic control		p-value
	Good (n = 65) Mean ± (SD)	Poor (n= 60) Mean ± (SD)	
White blood cell indices			
WBC (10^3/μL)	8.8191 (3.42811)	8.942 (2.59072)	0.074
Neu (10^3/μL)	6.0294 (4.51587)	5.966 (2.73258)	0.003
Lym (10^3/μL)	2.0745 (0.92139)	2.074 (0.73678)	0.173
Mon (10^3/μL)	0.5255 (0.12305)	0.665 (0.22931)	0.001
Eos (10^3/μL)	0.1689 (0.09584)	0.252 (0.023220)	0.000
Bas (10^3/μL)	0.0208 (0.00692)	0.03 (0.01913)	0.000
Red blood cell indices			
RBC (10^6/μL)	4.5211 (0.80951)	4.4460 (0.66449)	0.002
Hgb (g/dL)	11.7585 (1.64904)	12.30 (1.63168)	0.582
Hct (%)	37.0277 (4.75695)	37.74 (5.71567)	0.492
MCV (fL)	82.7938 (5.34491)	85.2 (7.62916)	0.092
MCH (Pg)	26.2692 (2.06003)	27.85 (2.42540)	0.187
MCHC (g/dL)	31.7169 (1.18580)	32.67 (0.91212)	0.003
RDWSD (fL)	43.4446 (5.71929)	43.14 (7.36771)	0.422
RDWCV (%)	14.2985 (1.89296)	14.14 (2.33566)	0.536
Platelet indices			
PLT (10^3/μL)	273.7385 (82.08385)	312.8 (79.94040)	0.045
PDW (fL)	12.9369 (3.49118)	12.41 (1.30042)	0.000
MPV (fL)	10.3185 (1.52417)	10.63 (0.62173)	0.000
PLCR (%)	27.5538 (12.00644)	30.07 (5.22135)	0.000
PCT (%)	0.2568 (0.04247)	0.335 (0.09527)	0.002
FBG (mg/dL)	135.0769 (10.73445)	186.3 (40.62908)	0.000

3.3. Correlation of Hematological Parameters and FBG in Patients with T2D

The current investigation employed the Pearson correlation examination to explore the correlation between fasting blood glucose and hematological indices in diabetics group (Table 3). The results revealed that neutrophil count, MCV, MCH, MCHC, PDW, MPV, PLCR, and PCT exhibited a statistically significant positive correlation with FBG. In contrast, Lym, RBC count, and Hct demonstrated a statistically significant negative correlation with FBG in T2DM.

Table 3. Correlation of hematological indices and FBG in diabetics group in a hospital in Fallujah, Iraq, from 1st April to 30th July 2023 (n=125).

Variables	FBG Correlation coefficient (r)	p-value
RBC (10^6/µL)	-0.180*	0.045
Hgb (g/dL)	-0.120	0.184
Hct (%)	-0.248**	0.005
MCV (fL)	0.527**	0.000
MCH (Pg)	0.469**	0.000
MCHC (g/dL)	0.207*	0.021
RDWSD (fL)	0.016	0.856
RDWCV (%)	-0.154	0.086
WBC (10^3/µL)	0.140	0.120
Neu (10^3/µL)	0.181*	0.043
Lym (10^3/µL)	-0.264**	0.003
Mon (10^3/µL)	0.062	0.494
Eos (10^3/µL)	0.021	0.814
Bas (10^3/µL)	0.128	0.154
PLT (10^3/µL)	-0.121	0.179
PDW (fL)	0.202*	0.024
MPV (fL)	0.358**	0.000
PLCR (%)	0.283**	0.001
PCT (%)	0.427**	0.000

**. Correlation has significant at 0.01
*. Correlation has significant at 0.05

4. DISCUSSION and CONCLUSION

In this study, it has been recorded that individuals who are diagnosed with diabetes have marked abnormalities in numerous hematological variables. The current study observed significant differences in both the mean and standard deviation of various blood parameters, including WBC, neutrophil count, lymphocyte count, basophil counts, Hct, MCHC, RDWSD, RDWCV, PLT, PDW, MPV, PLCR, and glucose levels, in a group of cases compared to a control population.

The current investigation achieved a significantly higher overall leukocyte count and absolute neutrophil count in diabetics. Similarly, papers reported in Bangladesh, northeastern Ethiopia, Libya, Turkey and northeastern Ethiopia among diabetics (Alam *et al*., 2015; Biadgo *et al*., 2016; Al Salhen & Mahmoud, 2017; Kizilgul *et al*., 2018; Arkew *et al*., 2021). The lymphocyte count and the basophil count were decreased a significant among diabetics. The previous researches confirm decreasing absolute basophil count in Bangladesh and Saudi Arabia in T2DM (Alam *et al*., 2015; Shehri, 2017). However, the absolute count of lymphocytes contradicts our findings, as surveys were conducted in Libya, India, and Nigeria (Al Salhen & Mahmoud, 2017; Harish *et al*., 2017; Awofisoye *et al*., 2019) reported mixed results.

The present study has revealed findings demonstrating a significant increase in both RDWSD and RDWCV. This study has yielded analogous with researchers in countries such as Saudi Arabia and Ethiopia (Shehri, 2017; Arkew *et al*., 2021). The elevated red RDW serves

as a reliable indicator of the presence of a diverse population of RBC circulating throughout the body, which is often associated with impaired erythropoiesis and erythrocytic dissociation (Salvagno *et al*., 2014) .Another explanation by Sherif *et al*., 2013, it is well known that chronic inflammation and high levels of oxidative stress, which are often seen in diabetes patients, may drastically impair the lifetime of RBC, which in turn can contribute to considerable variations in RBC volume.

Low MCHC and hematocrit levels were found in diabetics in the present study. Whereas MCHC and hematocrit were significantly low in diabetic patients, this finding is in line with what has been documented in India, Brazil, University of Gondar in Ethiopia, Northeast Ethiopia and (Farooqui *et al*., 2019; Knychala *et al*., 2021; Adane *et al*., 2021; Ebrahim *et al*., 2022). The current study found that the average PLT counts were lower in the cases group, whereas the average PDW, MPV, and PLCR were considerably higher. This report in harmony with other researchers such as Harish *et al*., 2017; Kizilgul *et al*., 2018; Ebrahim *et al.,* 2022. This might be due to persistent hyperglycemia, which is directly related to enzymatic glycosylation and increased expression of pro-inflammatory cytokines in the bloodstream (Barbieri *et al*., 2015; Bekele *et al*., 2019; Bhatt *et al*., 2020).

In this study, the mean Mon, Eos, Bas, MCHC, PLT, MPV, PLC-R, and PCT were a significant increasing in uncontrolled diabetes. Correspondingly, a plethora of congruous findings were documented in Southwest Ethiopia, Northeast Ethiopia, where the MPV and P-LC-R evinced a noteworthy escalation in patients with diabetes whose inadequate glycemic management (Asmamaw *et al*., 2021; Ebrahim *et al*., 2022).

On the contrary, it can be observed that there exists a significant decrease in the count of absolute neutrophils, red blood cells, and platelet distribution width among individuals with inadequate glycemic management. However, it is significant to mention that certain inconsistent results have been documented in India, wherein platelet distribution width exhibited a significant increase in patients with diabetes who had inadequate glycemic management (Farooqui *et al*., 2019). This finding is corroborated by further relevant studies conducted in India, Southwest, and Eastern Ethiopia, which reported a significant increase in red blood cell count among diabetes patients with inadequate glycemic control (Farooqui *et al*., 2019; Asmamaw *et al*., 2021; Arkew *et al*., 2022).

The current analysis, FBG has found a correlation with neutrophil count, MCV, MCH, MCHC, PDW, MPV, PLCR, and plateletcrit in individuals diagnosed with T2DM. This finding has confirmed by a paper was done in Ethiopia, demonstrating that levels of FBG rise in proportion to incremental increases in PDW, MPV, PLC-R, and plateletcrit (Ebrahim *et al*., 2022). Nevertheless, it is crucial to acknowledge that India has reported contradictory results, as MPV and MCV have shown an inverse relationship with FBG in individuals with diabetes (Joshi & Jaison, 2019). This study of type 2 diabetics discovered an inverse relationship between FBG and lymphocyte count, RBC, and Hct. This confirms the results of an earlier Ethiopian research that found the same thing among diabetics, there is an inverse relationship between FBG and RBC count and Hct (Ebrahim *et al*., 2022). However, it is important to note that this outcome contradicts the results of a study carried out in Japan, where RBC count was observed to be positively correlated with FBG in individuals with diabetes (Jaman *et al*., 2018).

This study showed a statistically significant effect of type 2 diabetes mellitus patients on some hematological parameters such as WBC, Hct, and MPV. Also, glycemic management has a significant correlation with hematological parameters such as RBC in patients with type 2 diabetes mellitus. Fasting blood glucose, it effects on hematological parameters among diabetics.

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. **Ethics Committee Number**: The present study was approved at 28/march/2023. Community Health/ Anbar Technical Institute/ Middle Technical University, Iraq, 00045.

Authorship Contribution Statement

Osamah Awad Ahmed: Supervision, Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Luay Awad Ahmed**: Methodology and Formal Analysis.

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REFERENCES

Adane, T., Asrie, F., Getaneh, Z., & Getawa, S. (2021). White blood cells and platelet profiles of diabetic patients at University of Gondar specialized referral hospital: A comparative cross-sectional study. *Journal of Clinical Laboratory Analysis*, *35*(6), e23808. https://doi.org/10.1002/jcla.23808

Agu, K.C. (2018). Diabetes mellitus: A review of some of the prognostic markers of response to treatment and management. *Journal of Insulin Resistance*, *3*(1), 1-10. https://doi.org/10.4102/jir.v3i1.36

Alam, J., Mallik, S.K., Hasan, M.J., Choudhury, S., & Sikder, H. (2015). A Comparative Analysis of Biochemical and Hematological Parameters in Diabetic and Non-Diabetic Adults. *Advanced Medical Sciences: An International Journal*, *2*(1), 1–9.

Alhadas, K.R., Santos, S.N., Freitas, M.M.S., Viana, S.M.S., Ribeiro, L.C., & Costa, M.B. (2016). *Are platelet indices useful in the evaluation of type 2 diabetic patients*?. *Jornal Brasileiro de Patologia e Medicina Laboratorial*, 52, 96-102. https://doi.org/10.5935/1676-2444.20160017

American Diabetes Association (2019). 6. *Glycemic targets: standards of medical care in diabetes-2019*. Diabetes Care, 42(suppl.1), 61–70.

American Diabetes Association (2014). Diagnosis and classification of diabetes mellitus. Diabetes care, 37(Supplement_1), S81-S90. https://doi.org/10.2337/dc19-S006

Antwi-Baffour, S., Kyeremeh, R., Boateng, S.O., Annison, L., & Seidu, M.A. (2018). Haematological parameters and lipid profile abnormalities among patients with Type-2 diabetes mellitus in Ghana. *Lipids in Health and Disease*, *17*(1), 1-9. https://doi.org/10.1186/s12944-018-0926-y

Arkew, M., Asmerom, H., Tesfa, T., Tsegaye, S., Gemechu, K., Bete, T., & Haile, K. (2022). Red blood cell parameters and their correlation with glycemic control among type 2 diabetic adult patients in Eastern Ethiopia: a comparative cross-sectional study. *Diabetes, Metabolic Syndrome and Obesity.Targets and Therapy*, *15*, 3499-3507. https://doi.org/10.2147/DMSO.S386093

Arkew, M., Yemane, T., Mengistu, Y., Gemechu, K., & Tesfaye, G. (2021). Hematological parameters of type 2 diabetic adult patients at Debre Berhan Referral Hospital, Northeast Ethiopia: A comparative cross-sectional study. *PloS one*, *16*(6), 0253286. https://doi.org/10.1371/journal.pone.0253286

Asmah, R.H., Yeboah, G., Asare-Anane, H., Antwi-Baffour, S., Archampong, T.N., Brown, C.A., & Ayeh-Kumi, P.F. (2015). Relationship between oxidative stress and haematological

indices in patients with diabetes in the Ghanaian population. *Clinical diabetes and endocrinology*, *1*(1), 1-5. https://doi.org/10.1186/s40842-015-0008-2

Asmamaw, M., Sime, T., Kene, K., Fekadie Baye, M., Teshome, M., & Zawdie, B. (2021). Evaluation of red blood cell parameters as a biomarker for long-term glycemic control monitoring among type 2 diabetic patients in Southwest Ethiopia: a cross-sectional study. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *14*, 4993–5000. https://doi:10.2147/DMSO.S348907

Awofisoye, O.I., Adeleye, J.O., Olaniyi, J.A., & Esan, A. (2019). Prevalence and correlates of anemia in type 2 diabetes mellitus: a study of a Nigerian outpatient diabetic population. *Sahel Medical Journal*, *22*(2), 55–63. https://doi.10.4103/smj.smj_65_18

Barbieri, J., Fontela, P.C., Winkelmann, E.R., Zimmermann, C.E.P., Sandri, Y.P., Mallet, E.K. V., & Frizzo, M.N. (2015). Anemia in patients with type 2 diabetes mellitus. *Anemia*, 1–7. https://doi.org/10.1155/2015/354737

Bekele, A., Teji Roba, K., Egata, G., & Gebremichael, B. (2019). Anemia and associated factors among type-2 diabetes mellitus patients attending public hospitals in Harari Region, Eastern Ethiopia. *PloS one*, *14*(12), 1-17.https://doi.org/10.1371/journal.pone.0225725

Bhatt, N., Dawande, P., Noman, O., Bahadure, S., & Bhake, A. (2020). Significance of hematological parameters in uncomplicated diabetes mellitus. *Indian Journal of Forensic Medicine & Toxicology*, *14*(4), 6279-6285. https://doi.org/10.37506/ijfmt.v14i4.12583

Biadgo, B., Melku, M., Abebe, S.M., & Abebe, M. (2016). Hematological indices and their correlation with fasting blood glucose level and anthropometric measurements in type 2 diabetes mellitus patients in Gondar, Northwest Ethiopia. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *9*, 91–99. https://doi.org/10.2147/DMSO.S97563

Çakırca, G., & Çelik, M.M. (2019). Relationships between glycemic control and platelet Indices, atherogenic index of plasma and vitamin D in patients with type 2 diabetes. *E Journal of Cardiovascular Medicine*, *7*(3), 147-52. https://doi.org/10.32596/ejcm.galenos.2019.08.047

Cappellini, M.D., & Motta, I. (2015). Anemia in Clinical Practice-Definition and Classification: Does Hemoglobin Change With Aging?. *Seminars in Hematology*, *52*(4), 261–269. https://doi.org/10.1053/j.seminhematol.2015.07.006

Ebrahim, H., Fiseha, T., Ebrahim, Y., & Bisetegn, H. (2022). Comparison of hematological parameters between type 2 diabetes mellitus patients and healthy controls at Dessie comprehensive specialized hospital, Northeast Ethiopia: Comparative cross-sectional study. *Plos One*, *17*(7), 0272145. https://doi.org/10.1371/journal.pone.0272145

Farooqui, R., Afsar, N., & Afroze, I.A. (2019). Role and Significance of Hematological parameters in Diabetes Mellitus. *Annals of Pathology and Laboratory Medicine*, *6*(3), 158–162. https://doi.org/10.21276/apalm.2355

Harish Kumar, S., Srinivasa, S.V., & Prabhakar, K. (2017). Haematological profile of diabetes and non-diabetes patients in rural tertiary center. *International Journal of Advances in Medicine, 4*(5), 1271–5. https://doi.org/10.18203/2349-3933.ijam20174111

International Diabetes Federation (IDF). (2021). IDF Diabetes Atlas. 10th Ed, 32-114.

Jabeen, F., Rizvi, H.A., Aziz, F., & Wasti, A.Z. (2013). Hyperglycemic induced variations in hematological indices in type 2 diabetics. *International Journal of Advanced Research*, *1*(8), 322–34.

Jaman, M.S., Rahman, M.S., Swarna, R.R., Mahato, J., Miah, M.M., & Ayshasiddeka, M. (2018). Diabetes and red blood cell parameters. *Annals of Clinical Endocrinology and Metabolism*, *2*, 1-009. https://doi.org/10.29328/journal.acem.1001004

Joshi, A.A., & Jaison, J. (2019). The Study of Platelet Parameters- Mean platelet volume (mpv) and platelet distribution width (PDW) in type 2 diabetes mellitus. *Annals of Pathology and Laboratory Medicine*, *6*(8), 407–413. https://doi.org/10.21276/APALM

Kaur, R., Kaur, M., & Singh, J. (2018). Endothelial dysfunction and platelet hyperactivity in type 2 diabetes mellitus: molecular insights and therapeutic strategies. *Cardiovascular Dialectology*, *17*(121), 1–17. https://doi.org/10.1186/s12933-018-0763-3

Kizilgul, M., Sencar, E., Bekir, U.C.A.N., Beysel, S., Ozcelik, O., Ozbek, M., & Cakal, E. (2018). Components of the complete blood count in type 2 diabetes mellitus with inadequate glycemic control. *Dicle Medical Journal*, *45*(2), 113-20. https://doi.org/10.5798/dicletip.410811

Knychala, M.A., Garrote-Filho, M.D.S., Batista da Silva, B., Neves de Oliveira, S., Yasminy Luz, S., Marques Rodrigues, M.O., & Penha-Silva, N. (2021). Red cell distribution width and erythrocyte osmotic stability in type 2 diabetes mellitus. *Journal of Cellular and Molecular Medicine*, *25*(5), 2505–16. https://doi.org/10.1111/jcmm.16184

Osman N.A., & Mansour M.M. (2013). *Measurement of some haematological parameters in diabetic patient attending military hospsital in Omdurman*. Sudan University of science and technology Institutional Digit Repos., 0–1.

Shehri, Z.S. (2017). The relationship between some biochemical and hematological changes in type 2 diabetes mellitus. *Biomedical Research and Therapy*, *4*(11), 1760-74. https://doi.org/10.15419/bmrat.v4i11.382

Al Salhen, K., & Mahmoud, A. (2017). Hematological profile of patients with type 2 diabetic mellitus in El-Beida, Libya. *Ibnosina Journal of Medicine and Biomedical Sciences*, *9*(03), 76-80. https://doi.org/10.4103/1947-489X.210115

Salvagno, G.L., Sanchis-Gomar, F., Picanza, A., & Lippi, G. (2014). Red blood cell distribution width: A simple parameter with multiple clinical applications. *Critical Reviews in Clinical Laboratory Sciences*, *52*(2), 86-105. https://doi.org/10.3109/10408363.2014.992064

Sherif, H., Ramadan, N., Radwan, M., Hamdy, E., & Reda, R. (2013). Red cell distribution width as a marker of inflammation in Type 2 diabetes mellitus. *Life Science Journal*, 10(3), 1501-1507.

Shin, G., Jang, K., Kim, M., Lee, J.H., & Yoo, H.J. (2020). Inflammatory Markers and Plasma Fatty Acids in Predicting WBC Level Alterations in Association with Glucose Related Markers: A Cross-sectional study. *Frontiers in Immunology*, *11*, 629. https://doi.org/10.3389/fimmu.2020.00629

World Health Organization (2016). *Global Report on Diabetes*. Geneva: World Health Organization; 6.

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Research Article

Carotenoid content, phytochemical screening, and antioxidant potential of Kantutay (*Lantana camara* L.), Katuray (*Sesbania grandiflora* L.), and Blue Ternate (*Clitoria ternatea* L.) flowers in the Philippines

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Abstract: Natural product chemistry plays a pivotal role in drug discovery and development. This study investigated the phytochemical profiles and antioxidant capacities of three prominent Philippine plants: Blue ternate (*Clitoria ternatea*), Kantutay (*Lantana camara*), and Katuray (*Sesbania grandiflora*). The primary aim is to provide a comprehensive assessment of their bioactive constituents and evaluate their potential for pharmacological applications. Phytochemical screening identified a diverse array of compounds, including flavonoids, tannins, glycosides, terpenoids, and other major chemical constituent classes, highlighting their therapeutic potential. The pigment analysis revealed substantial variations, with Blue ternate exhibiting the highest concentration, suggesting it as a promising source of carotenoids. Thin Layer Chromatography (TLC) and chlorophyll analysis further revealed distinct compound profiles. Total Phenolic Content (TPC) analysis and the DPPH radical scavenging method marked Blue ternate to have the highest phenolic content and the most potent antioxidant activity among the plant samples. These findings collectively emphasize the significant therapeutic potential of these plants, warranting further exploration for pharmaceutical development.

1. INTRODUCTION

The significance of plants in the natural world extends beyond their well-known roles in oxygen production, and carbon dioxide absorption, and as a vital source of sustenance for both animals and humans (Fernando, 2012). They also play a pivotal role in mitigating the greenhouse effect and addressing climate change (Fernando, 2012). However, the true depth of their importance lies in the remarkable diversity of natural products they produce, each holding its unique significance.

For thousands of years, plants and their chemical compounds have been intertwined with various cultures, serving as the foundation for an array of remedies and tonics (Veeresham, 2012). Even today, these natural compounds continue to captivate researchers, forming the bedrock of modern drug development (Masinas *et al.*, 2018). Extensive studies are dedicated

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to refining the safety profiles and exploring the efficacy of these compounds across various applications (Veeresham, 2012). Despite the introduction of groundbreaking technologies such as computer-based molecular modeling, combinatorial chemistry, and synthetic chemistry, the irreplaceable role of natural products in drug discovery and development remains (Fernando, 2012).

The advancements in technology, particularly in the realms of molecular biology and clinical observations, have ushered in a new era of understanding and application. However, the vast structural diversity found in many plant species leaves a wealth of untapped potential. These potential forms the basis of this study, which aims to give insight into three prominent plants found in the Philippines – Kantutay (*Lantana camara*), Katuray (*Sesbania grandiflora*), and Blue Ternate (*Clitoria ternatea*). This research focuses on determining carotenoid content, conducting a comprehensive phytochemical screening, and assessing the antioxidant capabilities of these plants.

Phytochemical screening is a method used to analyze the presence of phytoconstituents in a plant. This process starts with the preparation of aqueous and organic extracts (Srivastava *et al*., 2014). These extracts are obtained from plants that are rich in secondary metabolites (e.g., alkaloids, terpenes, flavonoids, etc.) and are commonly analyzed through thin-layer chromatography (TLC) (Purkait *et al*., 2023). The result of phytochemical screening depends on the morphology and structure of the plant. Moreover, analyzing the bioactive phytochemicals of a plant is substantial in determining its suitability for pharmacological development (Purkait *et al*., 2023).

Carotenoids are the pigments in many plants and vegetables that produce orange, red, and yellow hues (Healthline, 2017). Carotenoid contents of a plant mainly help in absorbing light and energy for photosynthesis. It has also an antioxidant function and hence acts as a type of antioxidant for humans which can fight certain types of cancer (Biology Dictionary, 2018; Szalay, 2015). Moreover, carotenoids are also viewed as safe chemicals for neutral chemical purposes and food supplementation due to their intense coloring abilities as well as their significance as precursors of Vitamin A (Hemalatha & Kailasam, 2022). It is composed of a long chain of alternating bonds and single C-C bonds which contain acid–functional groups, keto-hydroxyl-, and cyclic end groups (Hemalatha & Kailasam, 2022).

Plants are known as a rich source of antioxidants due to the many chemical components they contain, which may function alone or in concert to provide the body with defense against free radicals (Bhatt *et al*., 2013). These free radicals can cause oxidative stress which damages the cell and eventually results in some diseases like cancer, diabetes, eye disease, and age-related macular degeneration (Goodman *et al*., 2011). Given their significance, the three plants in this study will be analyzed to determine their bioactive components which will be of help in utilizing them for pharmacological developments in the Philippines and potentially in other countries.

Lantana camara, commonly known as Kantutay, belongs to the Verbenaceae family and is indigenous to the tropical and subtropical regions of the Americas (Kato-Noguchi & Kurniadie, 2021). This perennial herb or shrub can reach heights of 0.5-4.0 m and is characterized by its multicolored flowers, which can persist year-round in dense stands. While Kantutay's native habitat lies in the Americas, several of its taxa have been introduced and have become naturalized in Africa and tropical Asia (Negi *et al*., 2019). Kantutay encompasses an estimated 150 species, distributed across approximately 50 to 60 countries, with some being cultivated for their flowers (Munir, 1996).

Kantutay is classified as an invasive species in many regions, posing ecological challenges. Its rapid spread has disrupted terrestrial ecosystems and posed threats to agricultural areas by altering their structure (Kohli *et al*., 2006). Despite its invasive nature, Kantutay holds numerous medicinal applications. Experimental studies demonstrated its efficacy as an insecticidal, fungicidal, antimicrobial, and nematocidal agent (Begum *et al*., 2000). The bark of the Kantutay plant serves as an astringent and can be applied to leprous ulcers, while the leaves,

when they extracted, yield antimicrobial agents. Boiling the leaves and applying the solution alleviates body pain and swelling (Singh, 1996).

Furthermore, Kantutay leaves contain alkaloidal fractions that contribute to lowering blood pressure, stimulating intestinal movements, and enhancing respiration (Singh, 1996). Traditional uses for Kantutay extracts include addressing conditions like cancer, chicken pox, measles, rheumatism, tetanus, tumors, ulcers, high blood pressure, catarrhal infections, eczema, bilious fevers, and applying its oil for scabies and leprosy (Day *et al*., 2003; Ghisalberti, 2000; Sharma *et al*., 2007). A qualitative phytochemical screening conducted by Sardhara and Gopal (2013) in India revealed that Kantutay flowers contain a range of phytoconstituents including proteins, alkaloids, flavonoids, carbohydrates, sterols, tannins, terpenes, saponins, and glycosides. The ethanolic extract of Kantutay flowers was also analyzed, confirming the presence of flavonoids, terpenoids, ninhydrin, gelatin, phenols, and alkaloids. These identified phytochemicals underscore the pharmacological significance of Kantutay.

Sesbania grandiflora, commonly known as Katuray, belongs to the Leguminosae family and is endemic to Asia, including the Philippines, India, Indonesia, and Malaysia (Hasan *et al*., 2012). This perennial plant thrives in regions with an annual temperature range of 22-30°C and struggles in environments below 10°C. Katuray is known by various names, including Vegetable Hummingbird, West Indian Pea, Scarlet Wisteria, and Red Wisteria, but is predominantly referred to as Katuray in the Philippines. Katuray has a rich history of traditional use in folk medicine, particularly for its edible flowers (Kirtikar & Basu, 1995). The plant's flowers, roots, bark, leaves, and fruit or pod are all utilized for various medicinal applications. Katuray is described as a soft-wooded, short-lived, and quick-growing tree that can reach up to 12 meters in height (Wagh *et al*., 2009). Its flowers come in red and white varieties, and it produces elongated pods that hang vertically.

Unlike Kantutay, Katuray is not toxic and offers a wide range of medicinal benefits. In India, it has been traditionally used to address ailments like headaches, fevers, bronchitis, smallpox, rheumatism, inflammation, leprosy, gout, and anemia (Wagh *et al*., 2009). The plant also serves as a diuretic, emetic, laxative, and tonic in folk medicine. Notably, Katuray exhibits anxiolytic, anticonvulsive, and hepatoprotective properties (Wagh *et al*., 2009). The roots are powdered and mixed with water to create a poultice for body swelling, while the bark is employed for conditions like dysentery and sprue, acting as a laxative and emetic (Wagh *et al*., 2009). The leaves, when crushed or converted into juice, are used for various conditions including leprosy, fever, gout, and itchiness due to their anthelmintic and tonic properties. Additionally, Katuray flowers can be juiced and consumed to alleviate headaches, stuffy nose, and head congestion (Wagh *et al*., 2009).

Arun *et al*. (2014) conducted a phytochemical screening of Katuray leaves in India, identifying tannins, saponins, alkaloids, glycosides, cardiac glycosides, steroids, and flavonoids. Further studies confirmed the presence of valuable secondary metabolites, underscoring the medicinal potential of Katuray (Avalaskar *et al*., 2011; Gomase *et al*., 2012). Additionally, Katuray flowers were found to be rich in carotenoids, serving as a valuable source of Vitamin A and B9 (Bhokre *et al*., 2022).

Blue Ternate, scientifically known as *Clitoria ternatea*, belongs to the Fabaceae family and is recognized as a perennial herbaceous plant (Al-Snafi, 2016). Originating from the Ternate Island in Indonesia, it has spread to various Southeast Asian countries as well as other continents like Australia and America (National Nutrition Council, 2022). In the Philippines, it is commonly referred to as Blue Ternate but also goes by names such as Blue Bell Vine, Asian Pigeon Wings, Butterfly Pea, and Darwin Pea. Notably, the deep blue indigo color of its flowers adds to its ornamental value (NNC, 2022).

Blue Ternate holds both culinary and medicinal significance. In the Philippines, its flowers are frequently used to prepare herbal drinks and teas, and its natural pigment is employed as a food coloring agent for rice and bread, as well as a garnish for culinary. In addition, Blue

Ternate has a long history of traditional medicinal applications, particularly in Ayurveda. It has been utilized to address neurological disorders for centuries (Gollen *et al.*, 2018). The plant's roots are used for sore throats, abdominal enlargement, skin diseases, and ascites, while seeds and leaves are employed for memory enhancement. The flowers and their juice serve as an antidote for snake bites, and the seeds are applied for joint swelling, colds, and urinary issues (Ragupathy & Newmaster, 2009). Furthermore, there have been several reports of the pharmacological characteristics of Blue Ternate including sedative, anti-inflammatory, analgesic, anxiolytic, and antipyretic activities (Gollen *et al.*, 2018).

Phytochemical analyses have revealed the presence of a diverse array of compounds in Blue Ternate, including saponins, tannins, proteins, carbohydrates, triterpenoids, flavonoids, phenols, anthocyanins, flavanol glycosides, anthraquinone, volatile oils, cardiac glycosides, and Stigmast-4-ene-3,6-dione (Al-Snafi, 2016; Kelemu *et al.*, 2004). Additionally, the flower contains flavonoids, phenols, and anthocyanin phytoconstituents (Chayaratanasin *et al.*, 2015). These phytochemicals contribute to the plant's anti-inflammatory and analgesic properties (Srivastava *et al.*, 2009; Malik *et al.*, 2008). Notably, Blue Ternate flowers contain β-carotene, albeit in lower concentrations compared to some other plants, due to the prevalence of blue and violet hues associated with anthocyanins (Hemalatha & Kailasam, 2022).

In summary, the three plants hold immense botanical and medicinal significance in the Philippines and beyond. Their phytochemical compositions and biological activities offer a wealth of potential for various applications, ranging from traditional medicine to modern pharmacology. Further research and exploration of these plants' properties and compounds are essential for unlocking their full potential in the fields of health and medicine.

2. MATERIAL and METHODS

2.1. Sample Preparation

The three plant samples were collected and prepared at Pantabangan, Nueva Ecija, Philippines, 3124. The preparation of the samples for the extraction of phytochemicals and other natural products started with the air-drying at room temperature wherein the samples were placed in a room not directly hit by sunlight. The air-drying of samples lasted for 83 hours. Consequent to that, an osterizer was used to crush the plants into smaller pieces as shown in Figure 1. The crushed plant samples were extracted with ethanol (1 g plant material: 10 mL solvent) for 72 hours while being constantly stirred. By filtration, the extract and the residue were separated. With the use of a rotavapor drier (55-85℃), the extracts were dried and stored in glass bottles and centrifuge tubes coated with aluminum foil or paper and kept in the refrigerator until they were ready for analysis. Also, some grounded plant samples were kept in the fridge for pigment analysis.

Figure 1. Air dried (a) *Lantana camara*, (b) *Sesbania grandiflora*, and (c) *Clitoria ternatea*; (d) crushing the plant samples with osteorizer, (e) crushed plant sample subject for extraction, (f) and (g) are plant samples being extracted with ethanol subject for analysis.

2.2. Phytochemical Screening

In accordance with Pant *et al*. (2017) method, the extracts were subjected to qualitative phytochemical screening for the determination of the presence of the major chemical constituent classes (i.e., alkaloids, glycosides, carbohydrates, tannins, terpenoids, saponins, anthraquinones glycosides, cardiac glycosides, phenols, and flavonoids) through color reaction.

2.3. Pigment Analysis

2.3.1. *Total carotenoid analysis (TCA)*

The carotenoid analysis methodology was adapted from Natividad *et al.* (2014). After being placed in a 50 mL centrifuge tube, 500 mg of the plant powder was extracted with 10 mL of ethanol each using a vortex mixer for one minute. Measurements were taken of the supernatants. The carotenoid extract's ultimate volume was changed to 75 mL by adding 95% ethanol. The UV-Vis spectrophotometer was utilized to calculate the carotenoid extract's absorbance value at 450 nm. All plant samples underwent these procedures for three times each.

According to the following formula, the total carotenoid yield (dry weight) was determined:

$$\text{Total carotenoid yield}\left(\frac{\mu g}{g}\text{ dried weight}\right) = \frac{V(A - 0.0051)}{0.175W}$$

Where:

A – absorbance value of diluted extraction at 450 nm
V – final volume of the extract (mL)
0.175 – extinction coefficient of carotenoids
W – weight of the dried power (grams)

2.3.2. *Thin layer chromatographic (TLC) analysis*

With the use of a pencil, a baseline was drawn both at the bottom and top of a TLC distancing about 1 cm from the ends. Using a capillary tube, the sample extracts were applied to the TLC plate. The development chamber was a beaker with a watch glass lid and solvent within. The solvent mixture was poured into the development chamber after it had been lined with filter paper inside to ensure solvent saturation. Instead of using 5% methanol in toluene as the solvent, this experiment employed 5% methanol in xylene (Natividad & Rafael, 2014). Under the baseline of the TLC plates, more of the solvent mixture was injected into the chamber. Making sure that the baseline was above the solvent system, the TLC plate with spotted extracts was moved to the development chamber. It was noted how far the spot and solvent moved. An iodine chamber and an ultraviolet lamp were used to see the spots on the TLC plates. The spots were marked with a pencil (Velasco *et al*., 2018). Using the following formula, the retention factor (Rf) was calculated:

$$R_f = \frac{\text{distance travelled by the spot}}{\text{distance travelled by the solvent}}$$

To replicate the spots that were created, the TLC analysis was carried out three times.

2.3.3. *Chlorophyll analysis*

The method of Baluran and colleagues (2018a, 2018b) was used to determine the levels of chlorophyll in the samples of plants. Chlorophyll a and b concentrations of 2 mg/mL in methanolic plant extract solutions were measured at 666 and 653 nm using a UV-Vis spectrophotometer. The following formulas were used to determine the chlorophyll contents:

$$\text{Chlorophyll a}\left(\frac{mg}{L}\right) = 15.65\text{Abs}_{666} - 7.34\text{Abs}_{653}$$

$$\text{Chlorophyll b}\left(\frac{mg}{L}\right) = 27.05\text{Abs}_{653} - 11.21\text{Abs}_{666}$$

2.4. Total Phenolic Using Folin-Ciocalteau Reagent Determination

The Ortinero and colleagues' (2021) procedure was applied to analyze the total phenolic content (TPC) in the plant materials. 400 microliters of the extract, 1.0 mL of diluted Folin-Ciocalteau phenol reagent, and 800 µL of distilled water were added (1:10). 1.0 mL of 7.5% (w/v) sodium carbonate was mixed into the solution after waiting for five minutes. Several gallic acid standards were produced. With the use of a UV-Vis spectrophotometer, the absorbance of the extracts and the standard solutions were determined at 765 nm. The TPC of the plant extract was determined as milligrams of gallic acid equivalent per gram of the sample's dry weight (mg GAE/ g DW).

2.5. Determination of Antioxidant Activity Using DPPH Radical Scavenging Method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay) was carried out with a few minor adjustments using the procedure of Ortinero *et al*. (2021). The DPPH solution was made by mixing 6.0 mg of DPPH with 100 mL of methanol, whereas the extracts were produced in a range of solutions (0.01, 0.1, 1, 10, 100, and 1000 ppm). The test tube containing

2.5 mL of DPPH solution received precisely 1.5 mL of extract. BHA (beta hydroxy acids) served as the benchmark. After giving the mixture a vigorous shake, it was allowed to stand in the dark for 30 minutes. The UV-Vis Spectrophotometer 1500 was utilized to measure the solution's absorbance at 517 nm. The following equation was used to determine the DPPH radical scavenging activity of each extract:

$$\text{DPPH Scavenging activity} = \left(\frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}}\right) \times 100$$

The IC_{50} values, which were determined by using nonlinear regression (sigmoidal dose-response) on the data, represented the extracts' scavenging activity.

2.6. Data Analysis

Three copies of each experiment were run throughout. Analysis of variance (ANOVA) was used to compare the total phenolic, carotenoid, and chlorophyll contents of the plant samples at the 5% level of significance. The statistical analysis was completed utilizing Statistical Tool for Agricultural Research (STAR) software version 2.0.1.

3. RESULTS

3.1. Phytochemical Screening

The phytochemical screening of the Kantutay, Katuray, and Blue Ternate is shown in Table 1. The flowers of Blue Ternate contain tannins, glycosides, carbohydrates, cardiac glycosides, terpenoids, and phenol while proteins and saponins were absent in the Blue Ternate. The test for alkaloids for Blue Ternate was found positive on Mayer's reagent but negative on Dragendroff's reagent, which is similar to Kantutay. Moreover, the Blue Ternate contained flavonoids as it was found positive on both the Shinoda and Alkaline reagent tests. The Kantutay and Katuray contained all the natural products aside from proteins and phenol. However, the test for alkaloids for Katuray was the opposite of the results for Blue Ternate and Kantutay, because it was found positive on Dragendroff's reagent and negative on Mayer's reagent.

Table 1. Phytochemical contents in the plant samples determined in various dissolving agents.

Phytochemical Screening	Blue Ternate	Kantutay	Katuray
Test for tannins	+	+	+
Test for alkaloids			
• Mayer's reagent	+	+	-
• Dragendroff's reagent	-	-	+
Test for glycoside	+	+	+
Test for Carbohydrates (Molisch's test)	+	+	+
Test for saponins	-	+	+
Test for cardiac glycosides	+	+	+
Test for flavonoids			
• Shinoda test	+	+	+
• Alkaline reagent test	+	+	+
Test for terpenoids (Salkowski's test)	+	+	+
Test for proteins	-	-	-
Test for phenol	+	-	-

(−) Absence of phytochemical compounds
(+) Presence of phytochemicals compounds

Comparing the phytochemical screening results to other literature, the researcher found out that the Kantutay has almost similar results to that of the experiment of Sardhara and Gopal (2013) and Gul *et al*. (2020). From their experiment, the Kantutay also contained alkaloids, flavonoids, carbohydrates, tannins, terpenes, saponins, and glycosides. Sardhara and Gopal (2013) and Gul *et al*. (2020) also revealed that the test for protein had turned positive, which is in contrast to the negative result in this analysis. Further, Avalaskar and co-workers (2011) revealed that Katuray has tannin, polyphenols, saponins, and flavonoids. Their result is almost similar to the findings in this analysis, aside from the negative response for phenol. Moreover, Dethe and colleagues (2013) concluded that Katuray flowers have a negative response to terpenoids and glycosides, which have a positive response in this analysis. For Blue Ternate, the work of Al-Snafi (2016) with reference to Kelemu *et al*. (2004), stated that the Blue Ternate flower contained saponins, tannins, proteins, carbohydrates, flavonoids, phenols, and cardiac glycosides. The results were all the same with the analysis responses aside from the absence of saponins and proteins.

Tannins, being water-soluble phenolics found in all three plant samples, have applications in Asian medicine, particularly against diarrhea, and as astringents and diuretics (Chung *et al*., 2010; Khanbabaee & Ree, 2001; Sieniawska & Baj, 2017). They exhibit bioactivity in both absorbable and unabsorbable forms, influencing various organs systemically or targeting the gastrointestinal tract as antiviral, antimicrobial, and antioxidant agents (Navarro *et al*., 2018; Serrano *et al*., 2009).

Alkaloids, derived from amino acids, are detected in Blue Ternate and Kantutay with Mayer's reagent, and in Katuray with Dragendroff's reagent. Alkaloids are widely recognized in pharmacology for their antihypertensive, antiarrhythmic, antimalarial, and anticancer properties (Roberts & Wink, 1998).

Flavonoids, present in all three plant samples, have drawn interest in pharmacology due to their vitamin-like properties. They find applications in cosmetics, nutraceuticals, medicines, and pharmaceuticals, with demonstrated effects on cellular enzyme activity, as well as anti-carcinogenic, anti-mutagenic, anti-inflammatory, and antioxidative properties (Brandi, 1992; Panche *et al*., 2016).

Kantutay and Katuray contain saponins, which are increasingly valued for their diverse biological, pharmaceutical, and medicinal applications. Saponins exhibit anti-ulcer, adjuvant, anti-tumor, anti-inflammatory, hepatoprotective, and antibacterial properties (Moghimipour & Handali, 2014; El Aziz *et al*., 2019).

Terpenoids, present in all three samples, offer the potential for developing medications with fewer side effects. They are commonly found in essential oils and serve as flavoring and fragrance agents in the food industry. The pharmacological applications of these plants are extensive, as terpene-containing plants have been employed in traditional medicine for centuries, with various formulations available on the market (Ludwiczuk *et al*., 2017).

Among the three plant samples, only the Blue Ternate contained phenolics. Natural bioactive chemicals known as phenolic compounds are common in plant tissues. They have demonstrated fascinating bioactivities, including antibacterial and antiproliferative activities and antioxidant and anti-inflammatory properties, which has sparked a lot of interest in their utilization by many businesses (Albuquerque *et al*., 2021).

3.2. Pigment Analysis

3.2.1. *Total carotenoid analysis (TCA)*

The total carotenoid (TC) content expressed in µg/g for the three plant samples is shown in Table 2. From the TC analysis results, the values ranged from 213.03 to 538.36 µg/g in which the Kantutay has the lowest TC value, followed by Katuray, while Blue Ternate has the highest TC content. This result indicates that the Blue Ternate shows great potential as a source of carotenoids.

The TC content of the plant samples showed significant differences from the Analysis of Variance (ANOVA) at a 95% level of confidence. Moreover, from Duncan's Multiple Range Test (DMRT), the Blue Ternate has a significant amount of TC, followed by Katuray, and Kantutay has the least significant amount.

Table 2. Total carotenoid content of Blue Ternate, Kantutay, and Katuray.

Plant Samples	Total Carotenoid (TC) Content (μg/g)
Blue Ternate	538.36 ± 1.99^{a}
Kantutay	213.03 ± 0.55^{c}
Katuray	416.31 ± 0.75^{b}

Means with the same letter are not significantly different

The work of Meléndez-Martínez and colleagues (2021) revealed that the white, yellow, and red flowers of Kantutay have a TC content of 64.782±0.420, 2056.065±7.148, and 304.721±0.183 μg/g dry weight, respectively. These values were made up mostly of carotenoids like phytoene, 9-*Cis*-Violaxanthin, Lutein, Violaxanthin, 9-*Cis*-Anteraxanthin, α-carotene, and β-carotene. From the TC analysis in this experiment, the Kantutay has a TC of 213.03 μg/g, which is comparable to the white and red flowers' TC content from the previous studies.

Janarny and colleagues (2021) determined the TC of Blue Ternate and Katuray which are quantified as 18.2±0.1 and 82.1±0.5 mg β-carotene/g, respectively. Moreover, Weerasinghe and Gunathilake (2020) analyzed the β-carotene concentration of Katuray and revealed that it has 252.24±4.18 μg/g dry weight. Also, Bhokre and co-workers (2022) presented a carotenoid analysis of Katuray flowers and leaves and quantified it to be 420 μg/100g and 3,120 μg/100g, respectively.

The computed TC for Blue Ternate, Kantutay, and Katuray are close to the values written in the literature. Although there may be some obvious differences among the values, Dias and co-workers (2008) stated that the TC content could vary in terms of quantity and quality due to the variety, morphology, and maturity of the species involved. This statement is supported by the book of Rodriguez-Amaya (2001) about the protocol in food carotenoid analyses. It was stated in the book that factors like climate, maturity, geographic location, and various processes employed in the products affect the value and concentration of its carotenoid.

3.2.2. *Thin layer chromatography analysis*

TLC analysis was employed to separate the organic compounds, following the methods of Natividad and Rafael (2014). Instead of 5% methanol in toluene, 5% methanol in xylene served as the solvent system. The spots on the TLC plates were visualized under UV light and an iodine chamber, then marked with a pencil to record the distances traveled for *Rf* value calculation (Figure 2).

In total, 10 spots were identified in Blue Ternate, 11 in Kantutay, and 9 in Katuray. The *Rf* values ranged from 0.00 to 1.00 for Katuray and Kantutay, and from 0.00 to 0.99 for Blue Ternate. The ascending order of *Rf* values in Table 3 indicates differing polarities, with a value of 0.00 signifying a very polar compound. Similar *Rf* values suggest potential shared compounds in the plant extracts, with only two instances observed.

In particular, *Rf* values of 0.76 and 1.00 were observed in both the extracts of Kantutay and Katuray. It was also observed that there were values that did not have much differences. For instance, *Rf* values (in respective order) of 0.9 and 0.10 for Blue Ternate and Katuray, 0.15 and 0.14 for Blue Ternate and Kantutay, 0.36 and 0.35, 0.45 and 0.46, and 0.56 and 0.57 for Kantutay and Katuray, and 0.86, 0.87, and 0.88 for the three plant samples.

The polar compounds, which are very affine to the polar silica gel in the TLC plate, could be classified as oxygenated carotenoids i.e., lutein, echinenone, zeaxanthin, antheraxanthin, and

spirilloxanthin (Casuga & Natividad, 2023; Paiva & Russell, 1999; Velasco *et al*., 2018). The aforementioned oxygenated carotenoids may be present in the extract of the plant samples. On the other hand, those compounds that do not have a strong affinity for the silica gel in the TLC plate were considered to be very affine with mobile phase that has a great portion of xylene, a nonpolar solvent. In this case, high *Rf* values could be an indication of the presence of carotenoid hydrocarbon (i.e., β-carotene) (Natividad & Rafael, 2014).

Table 3. *Rf* values of the three plant samples separated by TLC.

	Blue Ternate	Kantutay	Katuray
Rf values	0.00	0.00	0.00
	0.09	0.05	0.10
	0.15	0.14	
	0.20	0.27	0.24
	0.30	0.36	0.35
	0.42	0.45	0.46
	0.48		
		0.56	0.57
	0.73	0.76	0.76
	0.86	0.88	0.87
		0.95	
	0.99	1.00	1.00
Total Spots	10	11	9

Figure 2. TLC analysis of Blue Ternate, Kantutay, and Katuray.

3.2.3. *Chlorophyll analysis*

The methods of Baluran et al. (2018a, 2018b) were adapted in this analysis for the determination of the chlorophyll concentrations. With the use of an ultraviolet-visible (UV-Vis) spectrophotometer, the methanolic solutions of the extracts of the sample plants for chlorophyll *a* and *b* were analyzed and the results are shown in Table 4.

Table 4. Chlorophyll *a* and *b* concentration in the three plant samples.

Plant Sample	Chlorophyll *a* (mg/L)	Chlorophyll *b* (mg/L)
Blue Ternate	0.81 ± 0.01^{c}	1.08 ± 0.02^{c}
Kantutay	1.12 ± 0.02^{b}	2.22 ± 0.03^{b}
Katuray	2.16 ± 0.01^{a}	3.13 ± 0.01^{a}

Means with the same letter are not significantly different

Chlorophyll *a* and *b* are known as two major types of chlorophyll that can be found in a plant where both are necessary for photosynthesis (Panawala, 2017). The key distinction between the two pigments is that chlorophyll *a* is regarded as a major or primary photosynthetic pigment, whereas chlorophyll *b* is an accessory pigment that gathers energy and transfers it to chlorophyll *a* (Panawala, 2017; Udayangani, 2011). The chlorophyll *a* concentration was recorded as 0.81, 1.12, and 2.16 for Blue Ternate, Kantutay, and Katuray, respectively. While chlorophyll *b* content was recorded as 1.08, 2.22, and 3.13 for Blue Ternate, Kantutay, and Katuray, respectively. Among the three plant samples, the Katuray has the highest chlorophyll *a* and *b* content, followed by Kantutay, while Blue Ternate has the lowest.

From the ANOVA (Analysis of Variance) with 5% level of significance, the concentration of chlorophyll *a* and *b* in the plant samples has significant differences. Moreover, from its DMRT, Katuray has a significant amount of chlorophyll *a* and *b*, followed by Kantutay, and Blue Ternate has the least amount of both concentrations.

3.3. Determination of Total Phenolic using Folin-Ciocalteau Reagent

The TPC in the extracts of the plant samples, expressed in mg of gallic acid equivalent (GAE) per gram dry weight of the sample was determined by the methods of Ortinero and colleagues (2021). The results of this analysis are shown in Table 5. Blue Ternate has the highest TPC amounting to 407.50 mg GAE/g dry weight, followed by Kantutay with 206.04 mg GAE/g dry weight, and Katuray has the lowest TPC of 107.07 mg GAE/g dry weight.

Table 5. Total phenolic content (TPC) determined in the plant samples.

Plant Sample	TPC (mg GAE/g dry weight)
Blue Ternate	407.50 ± 1.69^{a}
Kantutay	206.04 ± 4.39^{b}
Katuray	107.07 ± 2.52^{c}

Means with the same letter are not significantly different

Torres and co-workers (2021) determined the TPC of Blue Ternate and quantified it as 3.95 mg GAE/100 g (equivalent to 395 mg/GAE/g) which is comparable to the findings in this analysis of 407.50 mg GAE/g. Further, from the work of Baessa and colleagues (2019), different extracts were used in their experiment which are the infusion, decoction, and tincture. From those extracts, the TPC of Katuray was recorded as 163±1.00, 188±2.10, and 162±4.00 mg GAE/g DW, which values are close to the results in the TPC determined in this study. Moreover, Manzoor *et al*. (2013) employed different extraction techniques (i.e., Magnetic stirring, ultrasonic magnetic stirring, microwave-assisted magnetic stirring) and solvent systems (100% methanol, 100% ethanol, 80% methanol, and 80% ethanol) to determine the TPC of Kantutay. As a result, Kantutay contained 8.28±0.25 to 52.34±1.56 mg GAE/100 g DW.

From the ANOVA with a 5% level of significance, the TPC determined in the extracts of plant samples has significant differences. Moreover, from its DMRT, Blue Ternate has a significant TPC, followed by Kantutay, and Katuray has the lowest.

3.4 Antioxidant Potential using DPPH Radical Scavenging Activity

The antioxidant potential activity of the three plant samples was determined and different solutions of the plant sample extracts were prepared and tested to record the half-maximal inhibitory concentration (IC50) from the dose-response curve. IC50 is the concentration of the extract that indicates the amount of drug or test sample needed to inhibit a biological process or to produce the effect by half. Therefore, the lower the IC50 value, the more potent the substance for inhibiting a specific biological or biochemical function (Aykul & Martinez-Hackert, 2016).

The antioxidant potential activity of the plant samples is shown in Table 6. Blue ternate has the lowest value of 48.33 mg/mL, followed by Katuray with a value of 113.98 mg/mL, while Kantutay has the highest amount. Since this was evaluated with IC50, it means that the most potent among the plant samples is Blue ternate. Kantutay, on the other hand, is the least potent.

Table 6. Estimates of the half lethal concentration (IC_{50}) of the DPPH scavenging activity of the plant sample extracts

Plant Sample	IC_{50} (mg/mL)
Blue Ternate	48.33^{c}
Kantutay	209.99^{a}
Katuray	113.93^{b}

Means with the same letter are not significantly different

4. CONCLUSION

This study provided a comprehensive insight into the bioactive components of Kantutay, Katuray, and Blue Ternate. Phytochemical screening revealed a diverse array of compounds including flavonoids, tannins, glycosides, carbohydrates, cardiac glycosides, terpenoids, and phenols in Blue Ternate. Interestingly, proteins and saponins were absent in this species. Kantutay and Katuray also exhibited a rich phytochemical profile, lacking only proteins and phenols. Additionally, the alkaloid tests displayed unique responses for each plant.

Carotenoid content analysis showed varying concentrations, with Blue Ternate displaying the highest levels, indicating its potential as a source of carotenoids. TLC analysis unveiled distinct spots, with only a few similarities between Kantutay and Katuray extracts, suggesting potential shared compounds. Furthermore, chlorophyll analysis demonstrated differential concentrations of chlorophyll *a* and *b* in the three plant samples. The antioxidant potential activity was determined using DPPH radical scavenging method and evaluated with half-maximal inhibitory concentration revealing that the Blue ternate is the most potent to inhibit a specific biological or biochemical function among the plant samples.

The results of this study provide valuable insights into the bioactive components and antioxidant potential of these three prominent Philippine plants. These findings hold significant implications for their potential utilization in pharmacological developments, contributing to the advancement of traditional and modern medicine in the country. Further research into the specific compounds and their potential applications is warranted to fully unlock the therapeutic potential of these plants.

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. **Ethics Committee Number**: Central Luzon State University ERC No. 2023-071.

Authorship Contribution Statement

Melanie D. Piedad: Investigation, Resources, Formal Analysis, and Writing Original Draft. **Lexter R. Natividad:** Visualization, Software, Methodology, Supervision, and Validation.

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REFERENCES

Albuquerque, B.R., Heleno, S.A., Oliveira, M.B.P., Barros, L., & Ferreira, I.C. (2021). Phenolic compounds: Current industrial applications, limitations, and future challenges. *Food & Function*, *12*(1), 14-29. https://doi.org/10.1039/D0FO02324H

Al-Snafi, E.A. (2016). Pharmacological importance of *Clitoria ternatea* – A review. *IOSR Journal of Pharmacy, 6*(3), 68-83. ISSN: 2319-4219.

Arun, A., Karthikeyan, P., Sagadevan, P., Umamaheswari, R., & Rex Peo, R. (2014). Phytochemical screening of *Sesbania grandiflora* (Linn). *International Journal of Biosciences and Nanosciences, 1*(2), 33-36.

Avalaskar, A.N., Itankar, P.R., Joshi, V.S., Agrawal, M., & Vyas, J. (2011). Phytochemical and TLC studies of ethanolic extract of *Sesbania grandiflora* (Fabaceae). *International Journal of Pharmacology and Technology Research, 3*(3), 1346-1349.

Aykul, S., & Martinez-Hackert, E. (2016). Determination of the half-maximal inhibitory concentration using biosensor-based protein interaction analysis. *Analytic Biochemistry, 508*, 97-103. https://doi.org/10.1016/j.ab.2016.06.025

Baessa, M., Rodrigues, M.J., Pereira, C., Santos, T., da Rosa Neng, N., Nogueira, J.M.F., Barreira, L., Varela, J., Ahmed, H., Asif, S., Boukhari, S.A., Kayani, W.K., Khawaja Shafique Ahmad, Zengin, G., A., Custódio, L. (2019). A comparative study of the in vitro enzyme inhibitory and antioxidant activities of *Butea monosperma* (Lam.) Taub. and *Sesbania grandiflora* (L.) Poiret from Pakistan: New sources of natural products for public health problems. *South African Journal of Botany, 120*, 146-156, https://doi.org/10.1016/j.sajb.2018.04.006

Baluran, S.M., Natividad, L.R., Fernando, S.I., & Velasco, R.R. (2018a). Determination of pigments, antioxidant potential and vitamin contents of Benguet pine (*Pinus kesiya*) pollen. *International Journal of Biology, Pharmacy, and Allied Sciences* (IJBPAS), *7*(9), 1643-1651. https://doi.org/10.31032/IJBPAS/2018/7.9.4517

Baluran, S.M., Velasco, R.R., & Natividad, L.R. (2018b). Analyses of pigments, antioxidants, vitamins and immunostimulatory effect of Khasi pine (*Pinus kesiya*) pollen in Nile tilapia (*Oreochromis niloticus* L.). *Ascendens Asia Journal of Multidisciplinary Research Abstracts, 2*(5), 26. https://ojs.aaresearchindex.com/index.php/AAJMRA/article/view/3958

Begum, S., Wahab, A., Siddiqui, B. S., & Qamar, F. (2000). Nematicidal constituents of the aerial parts of *Lantana camara*. *Journal of Natural Products*, *63*(6), 765-767. https://doi.org/10.1021/np9903548

Bhatt, I.D., Rawat, S., & Rawal, R.S. (2013). *Antioxidants in medicinal plants*. In: Chandra, S., LATA, H., Varma, A. (eds) Biotechnology for Medicinal Plants. Springer. https://doi.org/10.1007/978-3-642-29974-2_13

Biology Dictionary. (2018). Carotenoids. https://biologydictionary.net/carotenoids/

Bhokre, C., Gadhe, K., & Joshi, A. (2022). Assessment of nutritional properties of *Sesbania grandiflora* flower and leaves. *The Pharma Innovation Journal, 11*(6), 90-94.

Brandi, M. L. (1992). Flavonoids: biochemical effects and therapeutic application. *Bone and Mineral, 19*(S1), S3-S14. https://doi.org/10.1016/0169-6009(92)90861-7

Casuga, K.M.S., & Natividad, L.R. (2023). Comparative analysis of pigment contents, phytochemical constituents, and antioxidant properties of selected plants in the Philippines. *International Research Journal of Science, Technology, Education, and Management, 3*(4), 10-20.

Chayaratanasin, P., Barbieri, M.A., Suanpairintr, N., & Adisakwattana, S. (2015). Inhibitory effect of *Clitoria ternatea* flower petal extract on fructose-induced protein glycation and oxidation-dependent damages to albumin in vitro. *BMC Complement Alternative Medicine, 15*(1), 1-9. https://doi.org/10.1186/s12906-015-0546-2

Chung, K.T., Wong, T.Y., Wei, C.I., Huang, Y.W., & Lin, Y. (2010). Tannins and human health: a review. *Critical Reviews in Food Science and Nutrition, 38*(6), 421-464. https://doi.org/10.1080/10408699891274273

Day, M.D., Wiley, C.J., Playford, J., & Zalucki, M.P. (2003). *Lantana: current management status and future prospects.* Australian Centre for International Agricultural Research, Number 114054. https://doi.org/10.22004/ag.econ.114054

Dethe, U.L., Joshi, S.S., Desai, S.S., & Aparadh, V.T. (2013). Screening of bioactive compounds of *Sesbania grandiflora* and *Pistia stratiotes*. *Indian Journal of Advances in Plant Research*, *1*(1), 27-30.

Dias, M.G., Camões, M.F., & Oliveira, L. (2008). Uncertainty estimation and in-house method validation of HPLC analysis of carotenoids for food composition data production. *Food Chemistry*, *109*(4), 815-824. https://doi.org/10.1016/j.foodchem.2007.12.026

El Aziz, M.M.A., Ashour, A.S., & Melad, A.S.G. (2019). A review on saponins from medicinal plants: chemistry, isolation, and determination. *Journal of Nanomedicine Research, 7*(4), 282-288. http://dx.doi.org/10.15406/jnmr.2019.07.00199

Fernando, W.G. (2012). Plants: An international scientific open access journal to publish all facets of plants, their functions and interactions with the environment and other living organisms. *Plants*, *1*(1), 1-5. https://doi.org/10.3390/plants1010001

Garcia, N.G., Daigdigan, V.D., Landingin, E.J., Garcia, G., & Natividad, L. (2023). Antibacterial Effect of Basil (*Ocimum basilicum*) and Table Salt as Alternative Disinfectants against *Staphylococcus aureus*. *Mindanao Journal of Science and Technology, 21*(2), 140-152.

Ghisalberti, E.L. (2000). *Lantana camara* L. (Verbenaceae). *Fitoterapia*, *71*(5), 467-486. https://doi.org/10.1016/S0367-326X(00)00202-1

Gollen, B., Mehla, J., & Gupta, P. (2018). *Clitoria ternatea* Linn: A herb with potential pharmacological activities: Future prospects as therapeutic Herbal Medicine. *Journal of Pharmacological Reports*, *3*(1), 141.

Gomase, P., Gomase, P., Anjum, S., Shakil, S., & Shahnvaj, K.M. (2012). *Sesbania sesban* Linn: A review on its ethnobotany, phytochemical, and pharmacological profile. *Asian Journal of Biomedical and Pharmaceutical Sciences*, *2*(12), 11-14.

Goodman, M., Bostick, R.M., Kucuk, O., & Jones, D.P. (2011). Clinical trials of antioxidants as cancer prevention agents: Past, present, future. *Free Radical Biology and Medicine*, *51*(5), 1068-1084. https://doi.org/10.1016/j.freeradbiomed.2011.05.018

Gul, H., Nisa, Z., Jabeen, A., Maryam, & Sajad, M.A. (2020). An evaluation of phytochemical screening of *Lantana camara* Linn. (An invasive plant in Pakistan). *Pure and Applied Biology*, *9*(3), 1856-1863. http://dx.doi.org/10.19045/bspab.2020.90198

Gundran, N.L., Ramirez, L., Rodolfo, A.W., Natividad, L., & Alili, R. (2018). Antimicrobial resistance and molecular detection of resistant genes in *Escherichia coli* isolates in poultry using polymerase chain reaction (PCR) assay. *Synergist*, 1, 26-31.

Hasan, N., Osman, H., Mohamad, S., Chong, W.K., Awang, K., & Zahariluddin, A.S.M. (2012). The chemical components of *Sesbania grandiflora* root and their antituberculosis activity. *Pharmaceuticals, 5*(8), 882-889. https://doi.org/10.3390/ph5080882

Healthline. (2017). Carotenoids: Everything you need to know. https://www.healthline.com/health/carotenoids

Hemalatha, T., & Kailasam, S.P. (2022). Carotenoid screening in selected flowers. *Journal of Xi'an Shiyou University, Natural Science Edition, 18*(12), 1160-1173.

Janarny, G., Ranaweera, K.K.D.S., & Gunathilake, K.D.P.P. (2021). Antioxidant activity of hydro-methanolic extracts of Sri Lankan edible flowers. *Biocatalysis and Agricultural Biotechnology,* 35, 102081. https://doi.org/10.1016/j.bcab.2021.102081

Khanbabaee, K., & Ree, T. (2001). Tannins: classification and definition. *Natural Product Reports*, *18*(6), 641-649. https://doi.org/10.1039/b101061l

Kato-Noguchi, H., & Kurniadie, D. (2021). Allelopathy of *Lantana camara* as an invasive plant. *Plants, 10*(5), 1028. https://doi.org/10.3390/plants10051028

Kelemu. S., Cardona, C., & Segura, G. (2004). Antimicrobial and insecticidal protein isolated from seeds of *Clitoria ternatea*, a tropical forage legume. *Plant Physiology and Biochemistry*, *42*(11), 867-873. https://doi.org/10.1016/j.plaphy.2004.10.013

Kirtikar, K.R., & Basu, B.D. (1995). *Indian medicinal plants* (2nd ed.). Bishen Singh and Mahendra pal singh, Allahabad, 2:084-1087.

Kohli, R.K., Batish, D.R., Singh, H.P., & Dogra, K.S. (2006). Status, invasiveness and environmental threats of three tropical American invasive weeds (*Parthenium hysterophorus* L., *Ageratum conyzoides* L., *Lantana camara* L.) in India. *Biological Invasion*, *8*, 1501-1510. https://doi.org/10.1007/s10530-005-5842-1

Ludwiczuk, A., Skalicka-Woźniak, K., & Georgiev, M.I. (2017). Terpenoids. *Pharmacognosy*, 233-266. https://doi.org/10.1016/B978-0-12-802104-0.00011-1

Malik, J.K., Manvi, F.V., Alagawadi, K.R., & Noolvi, M. (2008). Evaluation of anti-inflammatory activity of *Gymnema sylvestre* leaves extract in rats. *International Journal of Green Pharmacy*, *2*(2), 114-115. https://doi.org/10.22377/ijgp.v2i2.42.

Manzoor, M., Anwar, F., Sultana, B., & Mushtaq, M. (2013). Variation in antioxidant and antimicrobial activities in *Lantana camara* L. flowers in relation to extraction methods. *Acta Scientiarum Polonorum Technologia Alimentaria, 12*(3), 283-294. https://pubmed.ncbi.nlm.nih.gov/24584957/.

Masinas, K., Reyes, R.F., Iglesi, P.I., Natividad, L., & Merculio, J. (2018). Pharmaceutical Grade Pectin from Chinese Honey Orange (*Citrus poonensis*) and Dalanghita (*Citrus nobilis*) Peelings. *Synergist*, *1*, 62-67.

Meléndez-Martínez, A.J., Benítez, A., Corell, M., Hernanz, D., Mapelli-Brahm, P., Stinco, C., & Coyago-Cruz, E. (2021). Screening for innovative sources of carotenoids and phenolic antioxidants among flowers. *Foods*, *10*(11), 2625. https://doi.org/10.3390/foods10112625

Moghimipour, E., & Handali, S. (2014). Saponin: properties, methods of evaluation, and applications. *Annual Research and Review in Biology, 5*(3), 207-220. http://dx.doi.org/10.9734/ARRB/2015/11674

Munir, A.A. (1996). A taxonomic review of *Lantana camara* L. and L. *montevidensis* (Spreng.) Briq. (Verbenaceae) in Australia. *Journal of the Adelaide Botanic Garden*, *17*, 1-27.

National Nutritional Council. (2022). The health wonders of Blue Ternate. https://www.nnc.gov.ph/regional-offices/mindanao/region-ix-zamboanga-peninsula/8926-the-health-wonders-of-blue-Ternate

Natividad, L., Astrero, M.F., Basinga, L., & Calang, M.K. (2014). Effects of Different Systemic Insecticides in Carotenoid Content, Antibacterial Activity and Morphological Characteristics of Tomato (*Solanum lycopersicum* var *diamante*). *Asia Pacific Journal of Multidisciplinary Research, 2*(1), 209-213.

Natividad, L., & Rafael, R. (2014). Carotenoid Analyses and Antibacterial Assay of Annato (*Bixa orellana* L.), Carrot (*Daucus carota* L.), Corn (*Zea mays* L.) and Tomato (*Solanum lycopersicum* L.) Extracts. *Research Journal of Recent Sciences, 3*(3), 40-45.

Navarro, D.P., Del Rosario, C., Pangilinan, M.R. III, Natividad, L., & Cardenio, P. (2018). In Vitro Anthelmintic Property of *Indigofera zollingeriana* and *Leucaena leucocephala* Leaves Extracts against Gastro-Intestinal Nematodes in Goats (*Capra aegagrus hircus*). *Synergist*, *1*, 18 – 25. https://ssrn.com/abstract=4220485

Negi, G.C.S., Sharma, S., Vishvakarma, S.C.R., Samant, S.S., Maikhuri, R.K., Prasad, R.C., & Palni, L.M.S. (2019). Ecology and use of *Lantana camara* in India. *The Botanical Review, 85*, 109-130. https://doi.org/10.1007/s12229-019-09209-8

Ortinero, C., Rafael, R., Rayos, C.E., Bautista, K., Feliciano, M.A., Natividad, L., Natividad, G. (2021). Distilled Spirit Extraction of Phenolic Antioxidants from Fruit and Vegetable Residues. *Journal of Ecological Engineering*, *22*(1), 125-131. https://doi.org/10.12911/22998993/128864

Paiva, S.A.R., & Russell, R.M. (1999). β – carotene and other carotenoids. *Journal of the American College of Nutrition*, *18*(5), 426-433. https://doi.org/10.1080/07315724.1999.10718880

Panawala, L. (2017). *Difference between chlorophyll a and b*. PEDIAA. Available online: http://pediaa.com/difference-between-chlorophyll-a-and-b/

Panche, A.N., Diwan, A.D., & Chandra, S.R. (2016). Flavonoids: An overview. *Journal of Nutritional Science*, *5*, e-47. https://doi.org/10.1017/jns.2016.41

Pant, D.R., Pant, N.D., Saru, D.B., Yadav, U.N., & Khanal, D.P. (2017). Phytochemical screening and study of antioxidant, antimicrobial, antidiabetic, anti-inflammatory and analgesic activities of extracts from stem wood of *Pterocarpus marsupium* Roxburgh. *Journal of Intercultural Ethnopharmacology*, *6*(2), 170-176. https://doi.org/10.5455/jice.20170403094055

Purkait, M.K., Haldar, D., & Duarah, P. (2023). Chapter 2 – Utilization of various parts of the plant for extraction of phytochemicals and high-throughput screening techniques. *Advances in Extraction and Applications of Bioactive Phytochemicals,* 29-53. https://doi.org/10.1016/B978-0-443-18535-9.00004-1

Ragupathy, S., & Newmaster, S.G. (2009). Valorizing the 'Irulas' traditional knowledge of medicinal plants in the Kodiakkarai Reserve Forest, India. *Journal of Ethnobiology and Ethnomedicine*, *5*(10). https://doi.org/10.1186/1746-4269-5-10

Roberts, M.F., & Wink, M. (1998). *Alkaloids: Biochemistry, ecology, and medicinal applications*. Springer Science + Business Media, LLC. https://doi.org/10.1007/978-1-4757-2905-4

Rodriguez-Amaya, D.B. (2001). *A Guide to Carotenoid Analysis in Foods*. OMNI Research, ILSI Human Nutrition Institute, United States of America. Available online: https://pdf.usaid.gov/pdf_docs/Pnacq929.pdf

Sardhara, R.A., & Gopal, S. (2013). Qualitative phytochemical screening of different solvent extracts of *Tinospora cordifolia* stem and *Lantana camara* flower. *International Research Journal of Pharmaceutical and Applied Sciences*, *3*(5), 210-213.

Serrano, J., Puupponen-Pimiä, R., Dauer, A., Aura, A.M., & Saura-Calixto, F. (2009). Tannins: current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition & Food Research*, *53*(S2), S310-S329. https://doi.org/10.1002/mnfr.200900039

Sharma, O.P., Sharma, S., Pattabhi, V., Mahato, S.B., & Sharma, P.D. (2007). A review of the hepatotoxic plant *Lantana camara*. *Critical Reviews in Toxicology*, *37*(4), https://doi.org/10.1080/10408440601177863

Sieniawska, E., & Baj, T. (2017). Tannins. *Pharmacognosy*, 199-232. https://doi.org/10.1016/B978-0-12-802104-0.00010-X

Singh, S.P. (1996). *Biological control, in 50 years of crop*. Science Research in India; Indian Council of Agricultural Research: New Delhi, India, 88-116.

Srivastava, V., Khaan, S., & Banerjee, S. (2009). An evaluation of genetic fidelity of encapsulation microshoots of the medicinal plant: *Cineraria maritime* following six months of strong. *Plant Cell, Tissue and Organ Culture*, *99*(2), 193-198. http://dx.doi.org/10.1007/s11240-009-9593-z

Srivastava, P., Singh, M., Devi, G., & Chaturvedi, R. (2014). Chapter 30 – Herbal medicine and biotechnology for the benefit of human health. *Animal Biotechnology,* 563-575. https://doi.org/10.1016/B978-0-12-416002-6.00030-4

Szalay, J. (2015). What are carotenoids? *Live Science*. https://www.livescience.com/52487-carotenoids.html

Torres, R.C., Parcon, M.R.V., Esmundo, H.J.N., Canillo, D.C.P., & Ramil., C.C. (2021). Antioxidant activity and phytochemical constituents of Philippine *Clitoria ternatea* flowers as a potential therapeutic agent against infectious diseases. *Biological Sciences and Pharmaceutical Research, 10*(2), 12-18. https://doi.org/10.15739/ibspr.22.003

Udayangani, S. (2011). Difference between chlorophyll a and b. *Difference Between website*. https://www.differencebetween.com/difference-between-chlorophyll-a-and-vs-b/

Veeresham, C. (2012). Natural products derived from plants as a source of drugs. *Journal of Advanced Pharmaceutical Technology and Research*, *3*(4), 200-201. https://doi.org/10.4103/2231-4040.104709

Velasco, R.R., Dollente, D.J.M., Natividad, L.R., & Abella, T.A. (2018). Benguet Pine Pollen (*Pinus kesiya*) as natural source of Phytoandrogen. *International Journal of Biology, Pharmacy, and Allied Sciences* (IJBPAS), *7*(6), 1121–1132

Wagh, V.A., Wagh, K.V., Tandale, Y.N., & Salve, S.A. (2009). Phytochemical, pharmacological and phytopharmaceutics aspects of *Sesbania grandiflora* (Hadga): A review. *Journal of Pharmacy and Research, 2*(5), 889-892.

Weerasinghe, D., Upeka, K.A., Gunathilake, P., & Prasanna, K.D. (2020). Antioxidant properties of selected culinary flowers. *Annals: Food Science & Technology*, *21*(4), 783-791. https://afst.valahia.ro/wp-content/uploads/2022/09/IV.2_Upeka.pdf

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journal homepage: https://dergipark.org.tr/en/pub/ijsm **Research Article**

Selenium nanoparticles synthesized via green methods from Calluna vulgaris extract: Exploring their antioxidant and antibacterial activities

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Abstract: This study introduces a sustainable and environmentally friendly method for synthesizing selenium nanoparticles (SeNPs) by using Calluna vulgaris as a reducing agent. The process involves the addition of Na2SeO3 to a C. vulgaris aqueous solution, followed by reduction with ascorbic acid. UV-Vis spectroscopy confirmed SeNP formation, with a distinct absorption peak at 289 nm. Morphological analysis via Scanning Electron Microscopy (SEM) revealed spherical nanoparticles below 100 nm, as corroborated by Transmission Electron Microscopy (TEM) images displaying sizes ranging from 42.91 to 66.93 nm. Energy Dispersive Spectroscopy (EDS) confirmed the presence of selenium. Antibacterial assessments demonstrated the efficacy of *C.vulgaris* Selenium Nanoparticles (Cv-SeNPs) against gram-positive (*Enterococcus faecalis*, Staphylococcus aureus) and gram-negative bacteria (Escherichia coli). Cv-SeNPs exhibited notable antibacterial activity, particularly against E. Faecalis. In terms of antioxidant activities, Cv-SeNPs exhibited significant scavenging potential against DPPH and ABTS radicals, with low IC50 values of 24.72 and 16.87 µg/mL, respectively. The scavenging activities increased with concentration, reaching 86.6% for DPPH and 99.7% for ABTS at specific concentrations. The inclusion of ascorbic acid as a capping agent further augmented the free radical scavenging capabilities, indicating a synergistic relationship between selenium nanoparticles and capping agents. This research underscores the dual functionality of Cv-SeNPs as effective antibacterial agents and potent antioxidants. The green synthesis methodology utilizing C. vulgaris offers a sustainable approach for producing selenium nanoparticles with desirable characteristics, suggesting potential applications in medicine and industry. Further research on biomedical and industrial uses of Cv-SeNPs is needed.

1. INTRODUCTION

In recent years, nanotechnology has emerged as an incredibly promising and rapidly advancing field, owing to its extensive applications in applied sciences and technology (Yesilot & Aydin, 2019). Nanoparticles exhibit unique characteristics attributed to their high surface energy and significant surface-to-volume ratios. The popularity of metallic nanoparticles has soared due to their diverse applications across various scientific domains, encompassing physics, chemistry, materials science, and biomedical sciences (Raveendran *et al.,* 2003).

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Nanoparticles can be synthesized through three distinct methods: physical, chemical, and biological (commonly known as green synthesis). Physical methods necessitate expensive equipment, high temperatures, and high pressure. Chemical synthesis involves the use of toxic substances that pose environmental and health risks (Parveen *et al*., 2016). Additionally, these chemical methods are costly, toxic, and may be absorbed onto the nanoparticle surfaces (Adibian *et al.,* 2022). Due to the drawbacks associated with physical and chemical synthesis, there has been a shift towards green synthesis—an environmentally friendly and more cost-effective approach. Biological sources, such as plants, bacteria, fungi, and algae, containing natural molecules, are employed in green synthesis for nanoparticle production (Mittal *et al.,* 2013; Parveen *et al.,* 2016). This method offers a more sustainable and affordable alternative. Nanoparticles synthesized using plant extracts exhibit increased stability and a greater diversity in terms of shape and size compared to those produced by other organisms (Nazir *et al*., 2018).

Among the nanoparticles studied in recent years, SeNPs have attracted great attention due to their extraordinary physicochemical properties and potential biological activities (Barzegarparay *et al*., 2023). Selenium, an essential trace element, plays a vital role in maintaining human health and has been associated with a range of biological activities, including antioxidant and antibacterial properties (Roman *et al*., 2014; Mocchegiani *et al.,* 2009). The development of SeNPs from plant extracts offers a sustainable and biocompatible approach to harnessing the benefits of selenium (Khurana *et al*., 2019).

These nanoparticles show promising properties in the fields of medicine, biotechnology, and environmental science. Additionally, the production of selenium nanoparticles using natural resources (plant extracts and microorganisms) has attracted much attention by promoting sustainability and minimizing environmental hazards associated with traditional methods (Cittrarasu *et al*., 2021). One such natural source of interest for nanoparticle synthesis is *Calluna vulgaris*, commonly known as heather. Heather, a plant with widespread use in traditional medicine, is employed for treating a variety of conditions, including rheumatism, arthritis, eye diseases, kidney stones, inflammation of the bladder and kidneys, bronchitis, diarrhea, eczema, high blood pressure, increased irritability, anxiety, and sleep disorders. Extensive research has unveiled numerous pharmacological effects associated with heather, encompassing anti-inflammatory, antiseptic, sedative, diuretic, antiviral, cytotoxic, antiproliferative, antibacterial, cardioprotective, hepatoprotective, cytotoxic, and antioxidant properties (Kaunaite *et al*., 2022). *C. vulgaris* is an indigenous plant species found in many regions and has been recognized for its rich phytochemical composition, including flavonoids, phenolic acids, and other bioactive compounds (Starchenko *et al*., 2020). These compounds not only impart therapeutic properties to *C. vulgaris* but also serve as effective reducing and stabilizing agents for the green synthesis of nanoparticles (Mustapha *et al*., 2022; Shafey *et al*., 2020).

This study aims at the green synthesis of SeNPs using *C. vulgaris* extract as a reducing and capping agent. The utilization of *C. vulgaris* extract offers a sustainable and cost-effective approach, reducing the need for hazardous chemicals in the nanoparticle synthesis process. Furthermore, the phytochemicals present in *C. vulgaris* are expected to play a vital role in determining the properties and activities of the synthesized SeNPs. One of the primary objectives of this study is to investigate the antioxidant activity of SeNPs synthesized from *C. vulgaris* extract. Antioxidants play a crucial role in protecting cells and organisms from oxidative stress-induced damage by neutralizing harmful free radicals. Given the growing interest in natural antioxidants, understanding the potential of SeNPs synthesized from *C. vulgaris* extract as effective antioxidants holds substantial importance in the field of health and wellness. In addition to their antioxidant potential, SeNPs have also exhibited remarkable antibacterial properties. The development of effective antibacterial agents is crucial in combating the rising threats of antibiotic-resistant bacteria. Therefore, this study aims to evaluate the antibacterial activity of SeNPs synthesized from *C. vulgaris* extract against a range

of pathogenic bacteria, shedding light on their potential as an alternative or adjunctive therapy in bacterial infections.

2. MATERIAL and METHODS

2.1. Preparation of Heather (*C. vulgaris L.*) Extract

Dried heather (Ecodab, Batch no# P16S06) was obtained from a local market. The 5-gr weighed heather was ground into a powder using a mortar and pestle.100 mL of distilled water (dH_2O) was added to the powdered heather and mixed thoroughly. The mixture was boiled in a microwave oven for 1 minute (1200 W, 50 Hz). After cooling, the obtained extract was filtered through Whatman No. 1 filter paper to obtain the heather aqueous extract. For the synthesis of selenium nanoparticles, heather extract was freshly prepared and used.

2.2. Synthesis of Selenium Nanoparticles (Cv-SeNP)

In the production of selenium nanoparticles, the green synthesis method used by Wang *et al.* (2018) was modified and used. 20 mL of 50 mg/mL *C. vulgaris* extract was added dropwise to the prepared 80 mL of 10 mM Sodium Selenite (Na_2SeO_3) (Bostonchem, Boston, MA, Cas #10102-18-8) solution. The mixture was stirred at room temperature for 2 hours. Subsequently, 2 mL of freshly prepared L-Ascorbic acid (0.2 M) (Carlo Erba, France, Cas#50-81-7) was added dropwise to the mixture. Stirring was continued until a color change was observed.

2.3. Characterization of Selenium Nanoparticles

The UV-Vis spectra of the synthesized selenium nanoparticles were recorded using a Shimadzu UV-1801 UV-VIS spectrophotometer. The spectrum of the SeNP solution was measured in the wavelength range of 200-600 nm at 25°C. The particle size and morphology were observed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (JEOL JEM-1400 Plus). The elemental composition of the SeNP was determined using energy-dispersive X-ray spectroscopy (EDS) attached to the SEM (JEOL JSM-7100-F). The SEM, TEM, and EDS analysis were carried out at the Science and Technology Application and Research Center of Canakkale Onsekiz Mart University (COBILTUM).

2.4. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay

The antioxidant potential of selenium nanoparticles was assessed employing the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, following the procedure outlined by Saranya *et al.* (2023) with slight modifications. A 0.1 mM DPPH (abcr GmbH, Germany, Cas# 1898-66-4) solution was prepared using methanol. Selenium nanoparticles (SeNPs), synthesized at varying concentrations (0-5000 µg/mL), were mixed with the DPPH solution in a 1:1 ratio. Following the addition of the DPPH solution, the samples were left in the dark at room temperature for 30 minutes. Subsequently, the absorbance of each sample was measured at 517 nm using a UV-Vis spectrophotometer. DPPH served as the control, and methanol was employed as the blank. Ascorbic acid was utilized as the reference compound. IC_{50} values were obtained using GraphPad Prism software. The experiments were conducted in triplicate, and the free radical scavenging activity was expressed as a percentage of inhibition, calculated using the formula:

$$DPPH\ scavenging\ activity\ (\%) = \frac{(\text{Abs of control} - \text{Abs of sample})}{\text{Abs of control}} x100$$

2.5. ABTS (2,2-azinobis- (3-ethylbenzothiazoline-6-sulphonic acid)) Assay

The ABTS scavenging activity was determined by the method described by Re *et al.* (1999) with minor modifications. Briefly, ABTS* working solution was prepared by adding sodium persulfate (2.45 mM) to the prepared ABTS stock solution (7.4 mM) (PanReac Applichem GmbH, Darmstadt, Germany, Cas no#30931-67-0). To achieve an absorbance of 0.7 units at 734 nm, 1 mL of the ABTS* solution was diluted by combining it with 76 mL of methanol, as measured using a spectrophotometer. Selenium nanoparticles (500 µL) and control (methanol

(500 µL) were mixed with 500 µL of ABTS* solution and allowed to react. Absorbance was taken at 734 nm after 15 min using a spectrophotometer. IC_{50} values were obtained using GraphPad Prism software. Experiments were performed in triplicate and free radical scavenging activity was expressed as percent inhibition determined using the following formula:

$$ABTS\ scavenging\ activity\ (\%) = \frac{(\text{Abs of control} - \text{Abs of sample})}{\text{Abs of control}} x100$$

3. FINDINGS

3.1. Preparation and Characterization of Cv-SeNPs

An allotted volume of Na_2SeO_3 solution (10 mM) was introduced into an aqueous solution of *C. vulgaris* (50 mg/mL) and thoroughly mixed. Subsequently, a specific quantity of ascorbic acid was added to facilitate the reduction of the SeO_3^{2-} precursor to its atoms. Throughout the synthesis process, the augmented Se^o atoms aggregated into selenium nuclei, rapidly expanding as the redox reaction advanced, culminating in the generation of selenium nanoparticles (Wang *et al*., 2018). Following the reaction, a color change to ruby red was observed due to the formation of SeNP (Figure 1).

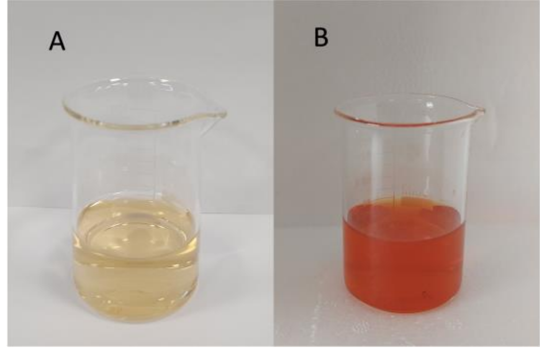

Figure 1. *C. vulgaris* extract **(A),** Selenium nanoparticles (Cv-SeNP) synthesized with *C. vulgaris* extract **(B).**

Along with these color changes, 200-600 nm wavelength scans were made in UV spectroscopy. UV-vis spectroscopy is an important method that reveals the formation and stability of SeNPs in aqueous solution by color change. Typically, the characteristic wavelength for synthesized selenium nanoparticles falls within the range of 200–300 nm (Barzegarparay *et al*., 2023; Zeebaree *et al*., 2020). By Uv-Vis measurement of Cv-SeNP, a shoulder absorption peak was observed at 289 nm, as illustrated in Figure 2. Similarly, few studies have reported the absorption peak of SeNPs to be approximately at 260-280 nm (Rajasekar & Kuppusamy, 2021; Shin *et al*., 2022; Gangadoo *et al*., 2017).

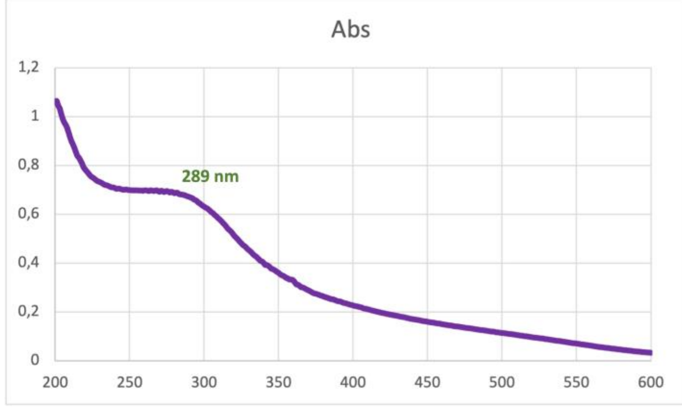

Figure 2. UV-Spectra analysis of SeNPs synthesized using *C. vulgaris* extract

The surface morphologies and sizes of SeNPs obtained by the green synthesis method were analyzed using SEM. The morphological analysis using SEM revealed that the nanoparticles exhibited a spherical structure with a size below 100 nm. The SEM image of SeNPs is shown in Figure 3A. EDS was used to determine the amount of elemental compounds and the purity of the nanoparticles. The highest peak in nanoparticles synthesized using *C. vulgaris* leaf extract was observed in selenium (15.2%) (Figure 3B). Other peaks observed with selenium in the EDS spectrum were carbon, oxygen, sodium, and very low amounts of copper and chlorine.

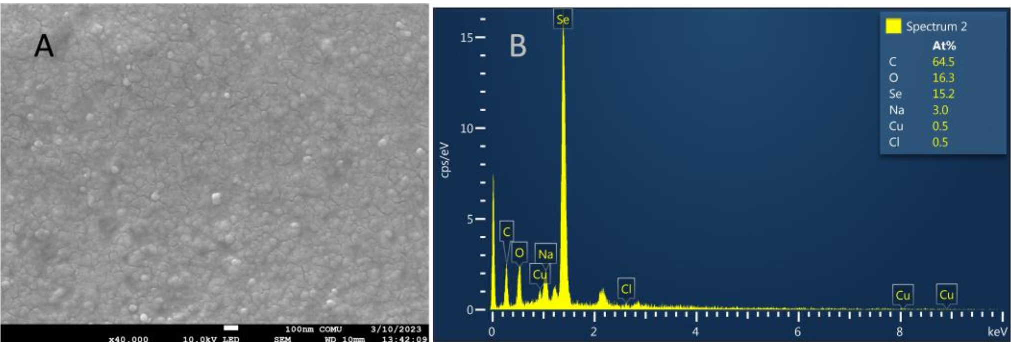

Figure 3. Scanning electron micrograph of synthesized Cv-SeNPs **(A)**, Identification of elemental composition using EDS spectra **(B)**

TEM images of Cv-SeNPs, as depicted in Figure 4, reveal a uniform spherical structure, aligning with the symmetrical single peak observed in the UV-Vis analysis. The sizes of the Cv-SeNPs range from 42.91 to 66.93 nm.

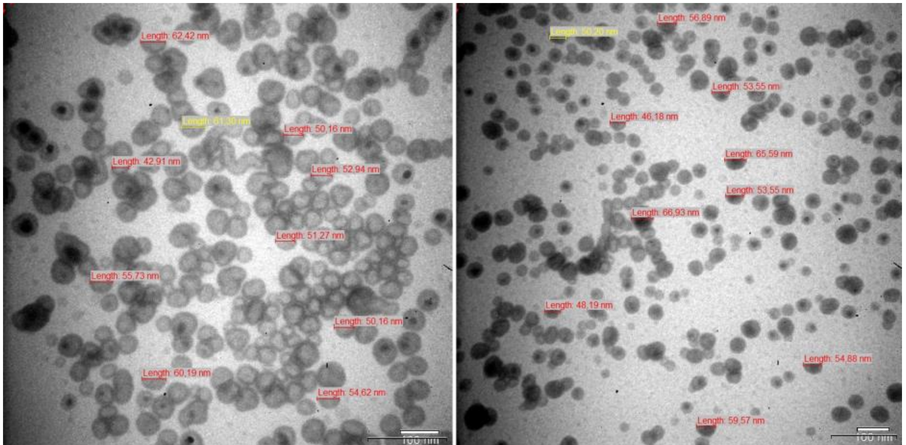

Figure 4. Transmission electron micrograph of synthesized Cv-SeNPs

3.2. Antibacterial Activity of Cv-SeNPs

In earlier research, various mechanisms for the antimicrobial effects of nanoparticles have been suggested by researchers. One crucial factor influencing this activity is the size of the nanoparticles. The small size allows nanoparticles to traverse cell walls and membranes, leading to cell lysis. Additionally, nanoparticles disrupt the respiratory cycle and the generation of ATP, hindering cell division, and ultimately resulting in the death of microbial cells (Zonaro *et al*., 2015). The antimicrobial activities of Cv-SeNPs were evaluated against three microorganism strains: gram-positive bacteria (*Enterococcus faecalis, Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*) by disk diffusion method. Additionally, a Penicillin/streptomycin double antibiotic disk was used as a positive control. It was determined that the positive control antibiotic showed a 22 mm diameter inhibition zone against the test microorganisms. It was observed that Cv-SeNPs synthesized by the green synthesis method were effective against all test microorganisms (Figure 5). Disc diameters are included when calculating the results. At a concentration of 1500 μg/mL, the antibacterial efficacy against the

three pathogenic microbes followed the order: *E. faecalis* > *S. aureus* > *E. coli*. The associated antibacterial zone diameters were 12.13 mm, 10.14 mm, and 10.09 mm, respectively.

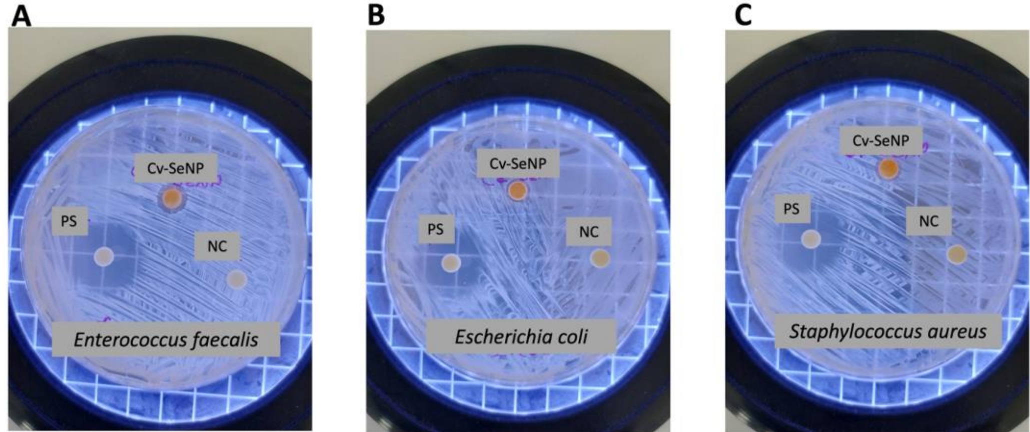

Figure 5. Antibacterial activity of Cv-SeNPs against the tested microorganisms **(A)** *Enterococcus faecalis,* **(B)** *Escherichia coli*, **(C)** *Staphylococcus aureus*

In previous studies, Se-NPs were found to have bactericidal properties against S. aureus (Shakibaie *et al*., 2015; Tran&Webster, 2011). Another study proved that Se-NPs have antibacterial properties against E. coli and S. aureus (Ali & Najmy, 2013). In a recent study, Se-NPs synthesized using Triphala extract exhibited significant antimicrobial activity against *S. mutans, S. aureus, E. faecalis*, and *Candida albicans* (Chellapa *et al*., 2020). SeNPs synthesized from *Solanum nigrum* fruit extract showed similar results against *S. typhi, E. coli, P. vulgaris*, and *V. cholerae* (Saranya *et al*., 2023).

3.3. Antioxidant Activity of Cv-SeNPs

The antioxidative potential of Cv-SeNPs was assessed using DPPH and ABTS* assays for scavenging free radicals. Cv-SeNPs exhibited significant antioxidant activity in both DPPH and ABTS assays, demonstrating IC_{50} values of 24.72 and 16.87 µg/mL, respectively. The low IC_{50} values highlight the effective neutralization of free radicals by Cv-SeNPs. Cv-SeNPs showed 86.6% maximum DPPH scavenging activity at a concentration of 750 µg/mL (Figure 6A). The results of the present study correlate well with the previous study in which the scavenging activity of *Olea ferruginea* fruit extract-mediated SeNPs was 85.2% (Hassan *et al*., 2022).

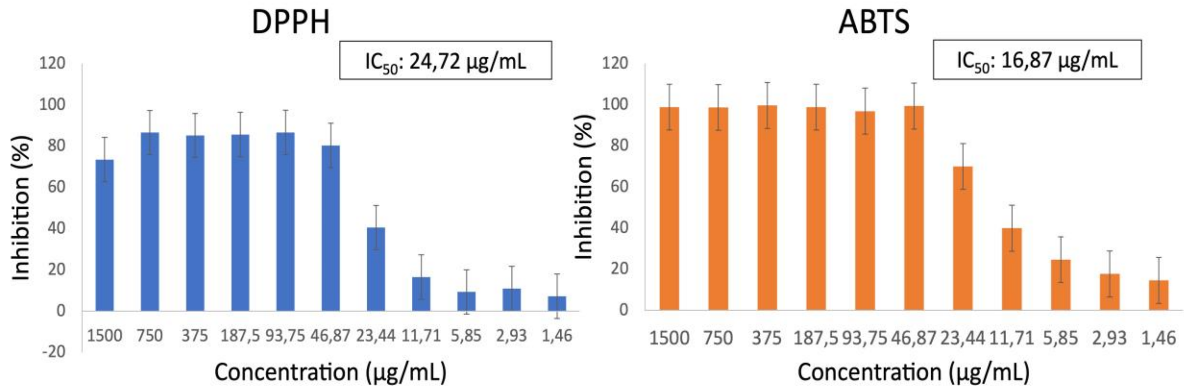

Figure 6. Antioxidant activity of the Cv-SeNPs **(A)** DPPH free radical scavenging, **(B)** ABTS radical scavenging activity

Figure 6B illustrates the antioxidant activity of SeNPs against ABTS*. Once again, the scavenging activity demonstrated an upward trend with increasing SeNP concentrations. At a concentration of 375 µg/mL, SeNPs exhibited a peak ABTS scavenging activity of 99.7%. These findings align with a prior study wherein the ABTS scavenging activity of SeNPs

mediated by *Rosa roxburghii* extract was reported as 98.92% (Ge *et al*., 2023), providing additional support for the results obtained in the current investigation. The incorporation of ascorbic acid as a capping agent for SeNPs enhanced the scavenging of free radicals, indicating a collaborative improvement in antioxidant capabilities. Selenium's capacity to counteract free radicals, primarily through the upregulation of selenoenzymes like glutathione peroxidase, is well-known (Rotruck *et al*., 1973; Shin *et al*., 2022). Introducing phytomolecules (such as phenolic and flavonoids) as capping agents further fortified selenium's antioxidant properties against free radicals. The findings suggest that the synergy between selenium nanoparticles and capping agents, including ascorbic acid and phytomolecules, results in potent antioxidants, demonstrating the potential to effectively address free radicals (Kokila *et al*., 2017; Gunti *et al*., 2019).

4. DISCUSSION and CONCLUSION

This research endeavors to shed light on the unique properties of SeNPs synthesized from *C. vulgaris*, their potential applications in medicine, and their contribution to the ever-growing field of green nanotechnology. *C. vulgaris*, a plant rich in phytochemicals, represents a promising source for the eco-friendly synthesis of SeNPs. This synthesis method not only reduces the environmental impact associated with traditional chemical methods but also provides an opportunity to explore the unique attributes of *C. vulgaris* in enhancing the biological activities of the synthesized nanoparticles.

Antioxidant activity is a critical parameter in the assessment of nanoparticles for potential biomedical applications. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defense mechanisms, is implicated in numerous diseases, including cancer, neurodegenerative disorders, and cardiovascular ailments. Selenium nanoparticles have demonstrated remarkable antioxidant potential due to their ability to scavenge ROS, thus holding promise as therapeutic agents in combating oxidative stress-related diseases.

Concomitantly, the antibacterial activity of SeNPs has attracted attention in the context of addressing the escalating global challenge of antibiotic resistance. As conventional antibiotics become less effective, the search for alternative antibacterial agents intensifies. Selenium nanoparticles exhibit antibacterial properties by disrupting the bacterial cell membrane, interfering with cellular functions, and promoting oxidative stress within bacteria. These attributes make SeNPs a potential solution to combat bacterial infections, both as standalone agents and in synergistic combinations with existing antibiotics.

In summary, this research bridges the domains of nanotechnology, green synthesis, and natural product chemistry, exploring the synthesis of SeNPs using *C. vulgaris* extract and their subsequent implications in antioxidant and antibacterial activities. The outcomes of this study may contribute to the development of novel therapeutic agents and environmentally friendly nanoparticle synthesis methodologies, fostering both health and sustainability.

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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

Ecem Erdem: Investigation, Methodology, Visualization, Writing – original draft. **Çiğdem Aydın Acar**: Investigation, Resources, Methodology, Visualization, Analysis, Supervision, Writing – review & editing.

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REFERENCES

Adibian, F., Ghaderi, R.S., Sabouri, Z., Davoodi, J., Kazemi, M., Ghazvini, K., Youssefi, M., Soleimanpour, S., & Darroudi, M. (2022). Green synthesis of selenium nanoparticles using *Rosmarinus officinalis* and investigated their antimicrobial activity. *BioMetals, 35,* 147–158. https://doi.org/10.1007/s10534-021-00356-3

Barzegarparay, F., Najafzadehvarzi, H., Pourbagher, R., Parsian, H., Ghoreishi, S.M., & Mortazavi-Derazkola, S. (2023). Green synthesis of novel selenium nanoparticles using *Crataegus monogyna* extract (SeNPs@ CM) and investigation of its toxicity, antioxidant capacity, and anticancer activity against MCF-7 as a breast cancer cell line. *Biomass Conversion and Biorefinery,* 1-10. https://doi.org/10.1007/s13399-023-04604-z

Cittrarasu, V., Kaliannan, D., Dharman, K., Maluventhen, V., Easwaran, M., Liu, W.C., Balasubramanian, B., & Arumugam, M. (2021). Green synthesis of selenium nanoparticles mediated from *Ceropegia bulbosa* Roxb extract and its cytotoxicity, antimicrobial, mosquitocidal and photocatalytic activities. *Scientific reports, 11*(1), 1032. https://doi.org/10.1038/s41598-020-80327-9

Gangadoo, S., Stanley, D., Hughes, R.J., Moore, R.J., & Chapman, J. (2017). The synthesis and characterisation of highly stable and reproducible selenium nanoparticles. *Inorganic and Nano-Metal Chemistry, 47*(11), 1568-1576. https://doi.org/10.1080/24701556.2017.1357611

Ge, Y.M., Xue, Y., Zhao, X.F., Liu, J.Z., Xing, W.C., Hu, S.W., & Gao, H.M. (2024). Antibacterial and antioxidant activities of a novel biosynthesized selenium nanoparticles using *Rosa roxburghii* extract and chitosan: Preparation, characterization, properties, and mechanisms. *International Journal of Biological Macromolecules, 254,* 127971. https://doi.org/10.1016/j.ijbiomac.2023.127971

Gunti, L., Dass, R.S., & Kalagatur, N.K. (2019). Phytofabrication of selenium nanoparticles from *Emblica officinalis* fruit extract and exploring its biopotential applications: antioxidant, antimicrobial, and biocompatibility. *Frontiers in microbiology, 10,* 931. https://doi.org/10.3389/fmicb.2019.00931

Hassan, H.U., Raja, N.I., Abasi, F., Mehmood, A., Qureshi, R., Manzoor, Z., Shahbaz, M., & Proćków, J. (2022). Comparative study of antimicrobial and antioxidant potential of *Olea ferruginea* fruit extract and its mediated selenium nanoparticles. *Molecules, 27*(16), 5194. https://doi.org/10.3390/molecules27165194

Kaunaite, V., Vilkickyte, G., & Raudone, L. (2022). Phytochemical diversity and antioxidant potential of wild heather (*Calluna vulgaris L.*) above ground parts. *Plants, 11*(17), 2207. https://doi.org/10.3390/plants11172207

Khurana, A., Tekula, S., Saifi, M.A., Venkatesh, P., & Godugu, C. (2019). Therapeutic applications of selenium nanoparticles. *Biomedicine & Pharmacotherapy, 111*, 802-812. https://doi.org/10.1016/j.biopha.2018.12. 146

Kokila, K., Elavarasan, N., & Sujatha, V. (2017). *Diospyros montana* leaf extract-mediated synthesis of selenium nanoparticles and their biological applications. *New Journal of Chemistry, 41*(15), 7481-7490. https://doi.org/10.1039/C7NJ01124E

Mittal, A.K., Chisti, Y., & Banerjee, U.C. (2013). Synthesis of metallic nanoparticles using plant extracts. *Biotechnology Advances, 31*(2), 346-356. https://doi.org/10.1016/j.biotechadv.2013.01.003

Mocchegiani, E., & Malavolta, M. (2009). Role of zinc and selenium in oxidative stress and immunosenescence: Implications for Healthy Ageing and Longevity. In: Fulop, T., Franceschi, C., Hirokawa, K., Pawelec, G. (eds) *Handbook on Immunosenescence: Basic*

Understanding and Clinical Applications, Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-9063-9_66

Mustapha, T., Misni, N., Ithnin, N.R., Daskum, A.M., & Unyah, N.Z. (2022). A review on plants and microorganisms mediated synthesis of silver nanoparticles, role of plants metabolites and applications. *International Journal of Environmental Research and Public Health*, *19*(2), 674. https://doi.org/10.3390/ijerph19020674

Nazir, S., Zaka, M., Adil, M., Abbasi, B.H., & Hano, C. (2018). Synthesis, characterization and bactericidal effect of ZnO nanoparticles via chemical and bio-assisted (*Silybum marianum* in vitro plantlets and callus extract) methods: a comparative study. *IET nanobiotechnology, 12*(5), 604-608. https://doi.org/10.1049/iet-nbt.2017.0067

Parveen, K., Banse, V., Ledwani, L. (2016). Green synthesis of nanoparticles: their advantages and disadvantages. *AIP Conf Proc., 1724*(1), 020048. https://doi.org/10.1063/1.4945168

Rajasekar, S., & Kuppusamy, S. (2021). Eco-friendly formulation of selenium nanoparticles and its functional characterization against breast cancer and normal cells. *Journal of Cluster Science, 32*(4), 907-915. https://doi.org/10.1007/s10876-020-01856-x

Raveendran, P., Fu, J., & Scott, L. (2003). Completely green synthesis and stabilization of metal nanoparticles. *J Am. Chem. Soc., 125,* 13940-13941. https://doi.org/10.1021/ja029267j

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine, 26*(9-10), 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Roman, M., Jitaru, P., & Barbante, C. (2014). Selenium biochemistry and its role for human health. *Metallomics, 6*(1), 25-54. https://doi.org/10.1039/c3mt00185g

Rotruck, J.T. [illegible]., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., & Hoekstra, W. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science, 179*(4073), 588-590.

Saranya, T., Ramya, S., Kavithaa, K., Paulpandi, M., Cheon, Y.P., Harysh-Winster, S., Balachandar, V., & Narayanasamy, A. (2023). Green synthesis of selenium nanoparticles using *Solanum nigrum* fruit extract and its anti-cancer efficacy against triple-negative breast cancer. *Journal of Cluster Science, 34*(4), 1709-1719. https://doi.org/10.1007/s10876-022-02334-2

Shafey, A.M.E. (2020). Green synthesis of metal and metal oxide nanoparticles from plant leaf extracts and their applications: A review. *Green Processing and Synthesis, 9*(1), 304-339. https://doi.org/10.1515/gps-2020-0031

Shin, S., Saravanakumar, K., Mariadoss, A.V.A., Hu, X., Sathiyaseelan, A., & Wang, M.H. (2022). Functionalization of selenium nanoparticles using the methanolic extract of *Cirsium setidens* and its antibacterial, antioxidant, and cytotoxicity activities. *Journal of Nanostructure in Chemistry, 12*(1), 23-32. https://doi.org/10.1007/s40097-021-00397-7

Starchenko, G., Hrytsyk, A., Raal, A., & Koshovyi, O. (2020). Phytochemical profile and pharmacological activities of water and hydroethanolic dry extracts of *Calluna vulgaris* (L.) Hull. herb. *Plants, 9*(6), 751. https://doi.org/10.3390/plants9060751

Wang, Y.Y., Qiu, W.Y., Sun, L., Ding, Z.C., & Yan, J.K. (2018). Preparation, characterization, and antioxidant capacities of selenium nanoparticles stabilized using polysaccharide–protein complexes from *Corbicula fluminea. Food Bioscience, 26,* 177-184. https://doi.org/10.1016/j.fbio.2018.10.014

Yesilot, S., & Aydin, C. (2019). Silver nanoparticles; a new hope in cancer therapy? *East J Med 24*(1), 111-116. https://doi.org/10.5505/ejm.2019.66487

Zeebaree, S.Y.S., Zeebaree, A.Y.S., & Zebari, O.I.H. (2020). Diagnosis of the multiple effect of selenium nanoparticles decorated by *Asteriscus graveolens* components in inhibiting HepG2 cell proliferation. *Sustainable Chemistry and Pharmacy, 15,* 100210. https://doi.org/10.1016/j.scp.2019.100210

Zonaro, E., Lampis, S., Turner, R.J., Qazi, S.J.S., & Vallini, G. (2015). Biogenic selenium and tellurium nanoparticles synthesized by environmental microbial isolates efficaciously inhibit bacterial planktonic cultures and biofilms. *Frontiers in Microbiology, 6,* 584. https://doi.org/10.3389/fmicb.2015.00584

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Research Article

Secondary metabolites of *Santolina africana*: chemical profiles and assessment of biological activities

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Abstract: Chemicals and antibiotics are serious problems that cause the resistance of bacteria and the persistence of chemical residues in food. These chemical products affect human health and promote diseases. Therefore, the use of natural resources, especially plants, appears as an alternative to avoid the harmful impacts of such products. Plant active substances such as essential oils, alkaloids and phenols are of great interest to scientists and have been studied for their biological activities. Essential oils (Eos) from the stems of *Santolina africana* were extracted by hydrodistillation and analyzed by Gaz Chromatography/ Mass Spectrometry (GC-MS). The antioxidant activity of crude extracts and Eos was evaluated by the DPPH assay and the antibacterial activity was evaluated by the disc diffusion method and the broth microdilution method against Gram-positive strains (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative strains (*Escherichia coli*, *Salmonella paratyphi,* and *Pseudomonas aeruginosa*). *S.africana* Eos from Morocco and Tunisia were found to be rich in artemisia ketone (35.4% and 44.3%, respectively), santolina alcohol (16.2%and 3.2%, respectively) and isoborneol (6.1% and 26.6%, respectively). Methanol extracts were rich in phenolic and flavonoids contents and showed the highest DPPH radical scavenging activity. Results exhibited the sensitivity of the strains to essential oils from *S. africana* especially against Gram-positive bacteria. This current research will provide new information about this plant that can be used as a natural antioxidant and antibacterial for industrial purposes.

1. INTRODUCTION

Since ancient times, plants have been used in food and traditional medicine. The use of herbal remedies for the prevention and treatment of diseases was often explained by their usage, since their biological activities were not yet proven. In recent years, the interest of scientists in plants has led to a systematic examination of bioactive molecules extracted from medicinal and aromatic herbs and plants. Scientific research demonstrated their pharmaceutical, medicinal and biological properties and exhibited their beneficial effects on health. Medicinal species have

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been shown to possess anti-inflammatory (Darwish *et al.*, 2020), antiviral (Javed *et al.*, 2021), anticancer (Dai *et al.*, 2010; Andrade *et al.*, 2018), antidiabetic (Béjaoui *et al.*, 2017), antimicrobial and antioxidant properties (Khammassi *et al.,* 2022). These properties are attributed to the bioactive compounds in essential oils (Eos) and phenolic compounds that are of wide interest in the pharmaceutical and food industries. Eos were complex mixtures of volatile secondary metabolites, including monoterpenes, sesquiterpenes and phenylpropanoids (Ismail *et al.*, 2014). They have been reported for their antioxidant, antifungal and antibacterial activities (Khammassi *et al.*, 2023). A special relevance was also gained to phenolic compounds that exhibited anti-inflammatory, antimicrobial, antioxidant and herbicidal properties (Khammassi *et al*., 2022; Khammassi *et al*., 2023).

S. africana (synonym: *Ormenis africana*) belongs to the Asteraceae family, is one of endemic species of the North of Africa (Tunisia, Algeria and Morocco). It is growing wild on the mountains and on the rocky slopes (Pottier Alapetite,1981). This plant is a green sub-shrubspecies with a strong odor, woody stems and yellow flowers. It is used in traditional medicine for its therapeutic effects. In Tunisia, it is used as an antidiabetic and for the treatment of colic and abdominal pain. A mixture of this plant and honey is used for the treatment of cardialgia ulcers (Ben Mansour, 2011; Bel Hadj Salah-Fatnassi, 2017). In Morocco, *S.. africana* is utilized as a stomachic, anthelmintic, abortifacientand vermifuge. It is also reported for its hypoglycemic effects (Lmachraa *et al*., 2014). Essential oils of *S. africana* have been reported to have antioxidant, antimicrobial (Boudjedjou, 2019), antidiabetic (Béjaoui *et al*., 2013) and antinflammatory activities (Malti *et al*., 2019). These volatile extracts have also been studied for their applicationas natural substances for agricultural control and plant protection. In fact, Eos from *S. africana* revealed fungicidal activity against phytopathogenic fungi (Khammassi *et al*., 2018). The chemical composition of the Santolina genus essential oils has been studied. Monoterpenes such as 1,8-cineole, myrcene, artemisia ketone and camphor were the main components of the volatile fractions of some Santolina species from different regions of the world (Tundis *et al*., 2018).

To our knowledge, few studies have been conducted on the chemical composition of *S. africana* essential oil from Tunisia and Morocco. Thus, the aims of this stuy were to investigate the variations in chemical composition of essential oils *S. africana* stems from the two countries and to evaluate the antioxydant and antibacterial activities of their volatile fractions and their crude extracts. Such study may provide natural alternative to chemical products and give solutions for the problems caused by antibiotics and synthetic antioxidants that affect human health.

2. MATERIAL and METHODS

2.1. Plant Material

Stems of *S. africana* were collected from the El Krib Sud region in the gouvernorate of Siliana (North of Tunisia) and from Taounate Province, located in the Fes region (North of Morocco). Ten samples were harvested from each country and identification was conducted by Dr Amri Ismail. A voucher specimen was deposed in the herbarium of INRGREF.

2.2. Essential Oils Extraction and Analysis

The essential oils (Eos) have been extracted from 100g of air-dried plant material by a Clevenger apparatus (European Pharmacopeia Method, 2008). Hydrodistillation has been conducted for 3hours. Volatile oils were stored at 4°C in darkglass bottles for analysis and the oil yields were calculated.

The composition of essential oils were determined by Gaz chromatography–Mass Spectrometry (GC-MS) analyses. GC/MS was performed on a Hewlett Packard 5972 MSD System. Eos separation was performed usinga HP-5 MS capillary column (30 m x 0.25 mm i.d., 0.25 mm film thickness) and helium as carrier gas (1.2 mL/min).

The GC-MS oven temperature was set at 50°C for 1 minute and then rose to 240°C at 5°C/min) and it remained at 204°C for 4 minutes.The injector and the detector were adjusted at temperatures of 250°C and 280°C, respectively. 0.1 mL of diluted samples (1%) were injected in splitless mode. ChemStation was the software used to process the mass spectra and chromatograms. Composition was identified based on mass spectra with comparison with Wiley spectra and Retention Index from alkanes (C9-C28 on the HP-5) helped in the identification of the chemical compounds.

2.3. Extract Preparation

50g of air-dried *S. africana* stems were powdered and extracted by maceration with distilled water or methanol. The extracts were filtered by filter paper (Whatman No. 4), evaporated with a rotary evaporator and then stored at 4°C for further analysis (Mau *et al.*, 2001).

2.4. Preliminary Phytochemical Screening

Extracts were dissolved in specific reagents through standard procedures and tested for phytochemical screening using standard methods (N'Guessan *et al.*, 2009; Karumi *et al.*, 2004; Dahoun, 2003).

2.5. Total Phenolic Content

The total phenolic content (TPC) was determined by the Folin-Ciocalteau assay using gallic acid as a standard phenolic compound according to the method described by Slinkard and Singleton (1977) and slightly modified by Dewanto *et al.* (2002). Briefly, 1 mL of each diluted extract was added to 1 mL of the Folin-Ciocalteu's phenol reagent and shaken. 3 minutes later, 1 mL of Na_2CO_3 (2 %) solution was added to the mixture. After incubation at room temperature for 90 minutes, absorbance against a reagent blank was read at 760 nm. The experiment was carried out in triplicates. TPCs were expressed as milligrams of gallic acid equivalents (GAE) per gram of weight (mg GAE/g DW).

2.6. Total Flavonoid Content

The total flavonoid content (TFC) was determined according to the method of chang *et al.,* (2002) by mixing 75 µL of $NaNO_2$ (7%) with 250µL of extract. 150µL of $AlCl_3$ (10%) and 500µL of NaOH (1 M) were then added to the mixture. After an incubation of 15 minutes at room temperature, the absorbance was read at 510 nm. Total flavonoids were determind using a calibration curve (50-600µg/mL) and presented in milligrams of quercetin equivalents per gram of dry weight (mg QE/g DW).

2.7. Determination of Radical-Scavenging Activity

The free radical-scavenging activity of each sample (essential oils/extracts) was evaluated with a DPPH (1,1-diphenyl-2-picrylhydrazil radical) assay (Wu *et al.*, 2006) with sligh modification. DPPH solution (10^{-4} M) was prepared in methanol. 1mL of each sample at different concentrations is added to 3 mL of the DPPH mixture, shaken and then allowed to sit for 30 minutes in the dark. The absorbance was then measured at 517 nm. Synthetic antioxidant butylatedhyroxytoluene (BHT) served as standard and assays were conducted in triplicate.

The percentage inhibition of DPPH radical was calculated according to the following formula:

$$\% \text{ Inhibition} = [(\text{A control} - \text{A sample})/\text{A control}] \text{ X } 100$$

The concentration providing 50% of the radical scavenging activity (IC_{50}) was then determined.

2.8. Antibacterial Activity of Essential Oils and Crude Extracts

The antibacterial activity was evaluated using the agar diffusion method (National Committee for Clinical Laboratory Standards NCCLS, 2003), against five bacteria strains; Gram-positive: *Bacillus subtilis, Staphylococcus aureus*; Gram-negative: *Salmonella paratyphi, Pseudomonas*

aeruginosa, *Escherichia coli*. The bacterial strains were grown on Muller Hinton medium (MHI) at 37°C for 24 hours.

100 µL of microorganism suspension adjusted to 10^6 CFU/mL was spread on petri dishes containing nutrient agar medium. Discs (8 mm of diameter) of sterile filter paper were then put in the plates and impregnated with 10 µL of essential oils or 100 µL of extracts. The petri dishes were incubated for 24hours at 37°C and inhibition zones (mm) were determined. The bacteria with a clear zone of inhibition of more than 12 mm were considered to be sensitive. For each test, the experiment was performed in triplicate. The antibacterial activity of Eos or extracts was compared with two antibiotics ;ampicillin (10 µg/disc) and spiramycin (10µg/disc).

2.9. Minimum Inhibitory (MIC) and Minimum Bactericide (MBC) Concentrations

The Minimum Inhibitory Concentration (MIC) was determined by the broth micro dilution method according to National Committee for Clinical Laboratory Standard-NCCLS (1999). Experiments were carried out in nutrient broth. Broth tubes containing 10^6 CFU/mL were filled with differentconcentrations of essential oils or extracts (1.5-7 mg/mL).Then, the samples were incubated using an incubator shaker to distribute the volatiles oils and subsequently examined for evidence of the growth. Each test was repeated in triplicate. DMSO in the broth tube served as a negative control.

When no visible growth was observed after incubation, the minimum inhibitory concentration (MIC) of the volatile oil or extract was determined. From tubes presenting no visible growth, 20 mL were spread on suitable nutrient agar petri dishes. The plates were then incubated for 24 hours. After subculturing, the lowest concentration of the essential oil or extract at which no visible growth was observed was considered as minimum bactericidal concentration (MBC).

2.10. Statistical Analysis

Analysis were conducted in triplicate and presented as average values. The data gained were subjected to a variance analysis (ANOVA) using SPSS software (Version 21.0) and analysed by means of the multiple comparison Student-Newman-Keuls test. Values of $p \leq 0.05$ were considered significantly different.

3. RESULTS and DISCUSSION

3.1. Phytochemical Analysis

Preliminary phytochemical screening of *S. africana* methanol (Me) and aqueous (Aq) extracts is reported to contain phenols, flavonoids, sterols, polyterpenes, tannins, and quinones. Saponins were found only in water extracts (Table 1) whereas results showed the absence of alkaloids in all the extracts. This result was not in accordance with those reported for plant extracts of the Asteraceae family. In fact, the aqueous and methanol extracts of *Chromolaena odorata* leaves showed the presence of alkaloids (N'Guessan *et al*., 2009). Plants contain different groups of phenolic compounds. Plant phenolics have gained considerable interestdue to their biological functions (Bouaziz*et al*., 2009).

Table 1. Phytochemical screening of *S.africana* crude methanol and aqueous extracts.

Extracts		Metabolites							
		Phenols	Flavonoids	Sterols and polyterpenes	Catechic tannins	Gallic tannins	Quinones	Saponins	Alkaloids
MeOH	Morocco	++	++	++	+	++	++	-	-
	Tunisia	++	++	++	+	++	+	-	-
Aqueous	Morocco	+	+	+	+	+	+	+	-
	Tunisia	+	+	+	+	+	+	+	-

++: strong; +: medium; - : poor (according to the color intensity). The measuring was repeated intriplicate.

3.2. Yields and Chemical Analysis

As shown in Figure 1, *S. africana* methanol extract yields were 9.12 % and 9.43 %, for Morocco and Tunisia, respectively. Whereas, aqueous extract yields did not exceed 1.71 %. Volatile oil yields of stems of *S. africana* from Tunisia and Morocco were 0.79 % and 0.9%, respectively. These yields are relatively in agreement with the data reported by Lmachraa *et al.,* (2014). However, they are lower when compared with the yield of *Santolina chamaecyparissus* var. *insularis* aerial parts (Poli*et al.*, 1997). The EO yield of stems from Algerian *S. africana* distilled in a Kaiser-Lang apparatus was 0.95 % (Zaiter *et al.*, 2015). Another study conducted on *S. africana* aerial parts from different locations in Algeria showed Eos yields ranging from 0.03 to 0.17 %. The variations in essential oil yields can be due to different factors such as plant parts, the date of harvest and environmental conditions. Moreover, Eos yiels can be affected by the physiological characteristics of the plants, genetic factors, soil type and methods of extraction (Saoud *et al*., 2013; Mohammad *et al*., 2022).

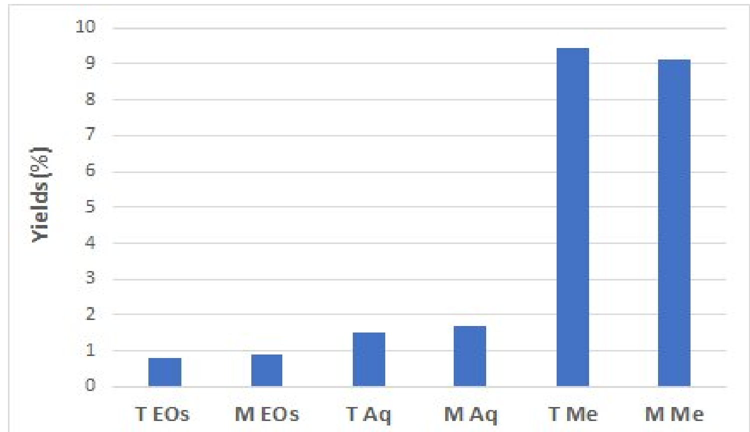


Figure 1. Yields of *S. africana* stems from Morocco and Tunisia.

T EO: Tunisian essential oil; M EO: Moroccan essential oil; T Me: Tunisian methanolic extract; M Me: Moroccan methanolic extract ; T Aq: Tunisian aqueous extract; M Aq: Moroccan aqueous extract

3.3. GC-MS Analysis

GC-MS analysis revealed a total of forty eight compounds, accounting 99.1 % and 98.9 % of the total Moroccan and Tunisian oils, respectively (Table 2 and Figure 2). Oxygenated monoterpenes (OM) were the main groups in *S. africana* Eos, followed by monoterpene hydrocarbons (MH). Sesquiterpene hydrocarbons (SH) ranged from 3.6% to 8.6%. However, previous investigations have demonstrated the dominance of hydrocarbon components in comparison with the oxygenated components in volatile fractions from other species of the Santolina genus (Zaiter *et al.,* 2015; Liu *et al.*, 2007).

Our results indicated that artemisia ketone was the most abundant in both essential oils, with variation in percentage between the two countries. Essential oil from Morocco was found to contain 35.4% artemisia ketone, 16.2 % santolina alcohol, 6.1 % isoborneol and 5.1 % β-oplopenone. Likewise, in Tunisian essential oil, artemisia ketone (44.3%), isoborneol (26.6%) and santolina alcohol (3.2 %) were identified as major compounds. In this study, the percentage and components of *S.africana* essential oil exhibited variation that can be caused by geographical origins (Díaz-Maroto *et al.*, 2006). Our findings were compared with other research involving other countries. It was reported that Eos extracted from aerial parts of *S.africana* in the flowering stage in three locations in the Eastern of Algeria (Batna province) were composed of germacrene D, spathulenol, myrcene, α-bisabolol, β-pinene, cys-chrysanhenol, 1,8-cineole, capillene, camphor santolina alcohol, lyratol and terpinen-4-ol (0.1-6.7 %) (Malti *et al.*, 2019).

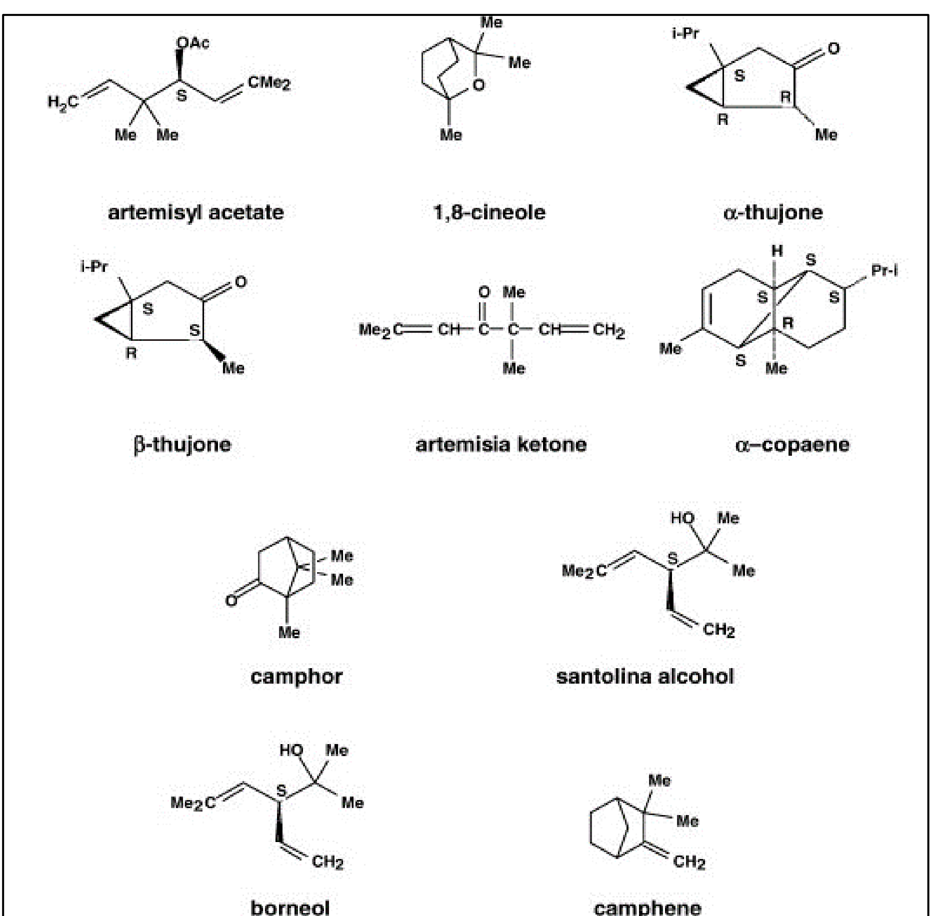
Table 2. Chemical profile of *S. africana* Eos from Tunisia and Morocco.

N°	Compounds	RI	Class	Formula	Area %		Identification
					Morocco	Tunisia	
1	Santolina triene	908	MH	$C_{10}H_{16}$	1.2	1.5	MS, RI
2	α-Tricyclene	926	MH	$C_{10}H_{16}$	0.2	1.1	MS, RI
3	α-Pinene	939	MH	$C_{10}H_{16}$	3.1	2.8	MS, RI, Co-In
4	Camphene	954	MH	$C_{10}H_{16}$	2.6	0.8	MS, RI
5	α-Sabinene	975	MH	$C_{10}H_{16}$	2.8	2.4	MS, RI
6	β-Pinene	979	MH	$C_{10}H_{16}$	4.7	2.9	MS, RI, Co-In
7	β-Myrcene	990	MH	$C_{10}H_{16}$	0.6	1.9	MS, RI, Co-In
8	α-Terpinene	1017	MH	$C_{10}H_{16}$	-	0.6	MS, RI, Co-In
9	*p*-Cymene	1024	MH	$C_{10}H_{14}$	0.5	0.8	MS, RI, Co-In
10	Santolina alcohol	1040	OM	$C_{10}H_{18}O$	16.2	3.2	MS, RI
11	Artemisia ketone	1062	OM	$C_{10}H_{16}O$	35.4	44.3	MS, RI
12	(*Z*)-Sabinene hydrate	1070	OM	$C_{10}H_{18}O$	1.1	0.3	MS, RI
13	α-Terpinolene	1088	MH	$C_{10}H_{16}$	0.3	0.9	MS, RI, Co-In
14	Linalool	1096	OM	$C_{10}H_{18}O$	3.6	0.2	MS, RI, Co-In
15	α-Thujone	1102	OM	$C_{10}H_{16}O$	0.8	-	MS, RI
16	β-Thujone	1114	OM	$C_{10}H_{16}O$	-	0.8	MS, RI, Co-In
17	*Trans*-Pinocarveol	1139	OM	$C_{10}H_{16}O$	0.6	-	MS, RI
18	*iso*-Menthone	1162	OM	$C_{10}H_{18}O$	-	0.6	MS, RI, Co-In
19	Isoborneol	1160	OM	$C_{10}H_{18}O$	6.1	26.6	MS, RI, Co-In
20	Terpinen-4-ol	1177	OM	$C_{10}H_{18}O$	1.2	-	MS, RI, Co-In
21	*p*-Cymen-8-ol	1182	OM	$C_{10}H_{14}O$	-	0.2	MS, RI
22	α-Terpineol	1188	OM	$C_{10}H_{18}O$	1.2	0.3	MS, RI, Co-In
23	Myrtenal	1195	OM	$C_{10}H_{14}O$	0.6	0.2	MS, RI, Co-In
24	(*E*)- Piperitol	1196	OM	$C_{10}H_{18}O$	0.5	0.2	MS, RI
25	(*Z*)-Carveol	1216	OM	$C_{10}H_{16}O$	0.2	0.3	MS, RI
26	Carvacrol methyl ether	1244	PP	$C_{11}H_{16}O$	0.8	0.4	MS, RI
27	α-Santalene	1417	SH	$C_{15}H_{24}$	1.3	-	MS, RI
28	(*Z*)-β-Farnesene	1442	SH	$C_{15}H_{24}$	0.6	-	MS, RI
29	α-Humulene	1454	SH	$C_{15}H_{24}$	0.5	0.6	MS, RI, Co-In
30	Allo-aromadendrene	1460	SH	$C_{15}H_{24}$	0.9	0.6	MS, RI
31	Germacrene D	1485	SH	$C_{15}H_{24}$	0.4	-	MS, RI, Co-In
32	Bicyclogermacrene	1500	SH	$C_{15}H_{24}$	0.8	-	MS, RI
33	α -Muurolene	1500	SH	$C_{15}H_{24}$	0.3	0.4	MS, RI
34	β-Bisabolene	1505	SH	$C_{15}H_{24}$	0.2	0.4	MS, RI
35	γ-Cadinene	1513	SH	$C_{15}H_{24}$	0.8	0.3	MS, RI
36	δ-Cadinene	1523	SH	$C_{15}H_{24}$	0.2	-	MS, RI
37	α-Cadinene	1538	SH	$C_{15}H_{24}$	0.2	-	MS, RI
38	β-Calacorene	1565	SH	$C_{15}H_{24}$	0.1	-	MS, RI
39	Germacrene B	1561	SH	$C_{15}H_{24}$	0.4	0.1	MS, RI
40	Germacrene D-4-ol	1575	OS	$C_{15}H_{26}O$	0.3	-	MS, RI
41	Spathulenol	1578	OS	$C_{15}H_{24}O$	0.9	1.2	MS, RI
42	Caryophyllene oxide	1583	OS	$C_{15}H_{24}O$	0.2	-	MS, RI
43	Guaiol	1600	OS	$C_{15}H_{26}O$	-	0.8	MS, RI
44	β-Oplopenone	1607	OS	$C_{15}H_{24}O$	5.1	-	MS, RI
45	1-epi-Cubenol	1628	OS	$C_{15}H_{26}O$	0.8	0.8	MS, RI
46	(*Z*,*E*)-Farnesol	1722	OS	$C_{15}H_{26}O$	0.2	0.4	MS, RI
47	(*Z*,*Z*)-Farnesol	1698	OS	$C_{15}H_{26}O$	0.2	-	MS, RI
48	(*E*,*E*)-Farnesol	1742	OS	$C_{15}H_{26}O$	0.4	-	MS, RI
Total identification					99.1	98.9	
Monoterpene hydrocarbons (MH)					16	15.7	
Oxygenated monoterpenes (OM)					67.5	77.2	
Phenylpropanoid derivates (PP)					0.8	0.4	
Sesquiterpenes hydrocarbons (SH)					8.6	3.6	
Oxygenated sesquiterpenes (OS)					6.7	2.4	

Another study conducted on Algerian *S.africana* EO (location Ichemoul, province of Betna) at the flowering stage, exhibited its richness in β-pinene, 1,8-cineole, germacrene D, sabinene, α-bisabolol and hedycaryol (Boudjedjou *et al.,* 2019). Similarly, Zaiter *et al.,* (2015), reported that the essential oil of the aerial parts of *S.africana* from Setif provence (North Eastern Algeria) is composed of β-pinene, myrcene and at less extent, α-pinene. Compared to Tunisian reports, there are notable differences with the oil of *S. africana* leaves collected from Siliana, a province in Tunisia, which is composed of artemisia ketone and isoborneol, presenting 42.96%, 24.30%, respectively (Khammassi, 2018).

In a previous study conducted on *S.africana* aerial part from Morocco (province of Tahanaout), oxygenated monoterpenes were particularly abundant (97.24 %) and camphor (54.3 %), borneol (17.24 %) and 1,8 cineole (5.27 %) were the main coumpounds (Lmachraa *et al.*, 2014).

The variation in the composition of volatile oil can be attributed to the geographical location of the plant and also to the plant parts (seeds, leaves and stems) which explain the aim of this investigation and also explain the exploitation of such bio resources for their biological properties.



RI: retention index; MS = mass spectrometry; Inj = co-injection with authentic compounds; - = absent

Figure 2. Main compounds of *S.africana* essential oil.

3.4. Total phenolic and Flavonoid Content

The results of total phenolic and flavonoids content are presented in Table 3. Statistical analysis revealed that the amounts of total phenolic content were significantly different between Me and aq extracts. In fact, Me extracts were richer in phenolics than aq extracts with amounts of 137.46 mg GAE/g DW and 137.03 mg GAE/g DW for Morocco and Tunisia. Flavonoids contents

differ significantly between extracts and methanol extracts exhibited their richness in flavonoids,with amounts ranging from 31.79 to 38.2 mg QE/g DW.Béjaoui *et al.*, (2013)reported that total phenolics and total flavonoid amounts were 50mg GAE/100g DM and 42.56 QE/g of dry mass, respectively, for the methanol extract of *S.africana* leaves collected from the region of ELKEF. Another study conducted by Ben Mansour *et al.*, (2011) exhibited that hydroethanolic extracts from inflorescences contain 312.07 ± 4.81 mg GAE/g dray matter and 73.72 ± 1.98 QE/g of dry mass of the phenolic and flavonoids compounds, respectively. The difference found between our results in comparison to other work may be due to the plant part used, solvent and the method of extraction. Previous research conducted on Asteraceae species showed high amounts of phenols (Wojdyło *et al.*, 2007).

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. The phenolic compounds may contribute directly to antioxidative action (Hatano *et al.*,1989).

Table 3. Total phenolic and flavonoid content of methanol and aqueous extracts of *S.africana.*

	Methanol extract		Aqueous extract	
	Morocco	Tunisia	Morocco	Tunisia
Total phenolic (mg GAE/g DW)	137.46± 0.14[a]	137.03± 0.31[a]	63.36±0.3[b]	59.34± 0.2[c]
Flavonoid content (mg QE/g DW)	38.2 ±0.15[a]	31.79± 0.22[b]	9.26±0.58[c]	7.57±0.21[d]

3.5. Antiradical Scavenging Activity

The Eos and crude extracts were screened for their antioxidant potential by DPPH assays. Statistical results showed that the radical scavenging activity differs significantly between extracts and origins (Figure 3). Methanol extracts were the strongest antioxidants followed by aqueous extracts and revealed high potential as compared with BHT. Eosexhibited the highest IC_{50}= 538.12 and 613.84 µg/mL for Moroccan and Tunisian species, respectively and then they presented the lowest antiradical activities. Research conducted on the antioxidant activity of the methanol extract leaves of *S. africana* showed significant antiradical potential towards DPPH radicals (46 µm Trolox equivalents TE) (Béjaoui *et al.*, 2013). Another study demonstrated the antioxidant activity of EO of *S. africana* aerial parts from Algeria with an IC_{50} value of 1.51 mg/mL (Malti *et al.*, 2019). The antioxidant activity of plant extracts can be attributed to their richness in total phenolics and flavonoids. Many reports showed a correlation between phenolic contents and the antioxidant properties of the plants. The chemical composition of bioactive compounds is one of the major factors that influences the activity of natural antioxidants (Bouaziz *et al.*, 2009; Shahidi & Marian, 2003). In fact, as plant secondary metabolites, the phenolics can react by different mechanisms: by inactivating lipid free radical chains, chelating redox-active metal ions, and avoiding hydroperoxide conversions into reactive oxyradicals. Radical scavenging by polyphenols is the most widely published mechanism for their antioxidant activity. In this radical scavenging mechanism, polyphenols sacrificially reduce reactive oxygen and nitrogen species ROS/RNS, such as •OH, O2•-, NO•, or OONO- after generation, preventing damage to biomolecules or theformation of a more reactive oxygen system (Perron*et al.*, 2009). While polyphenols are primarily recognized for their antioxidant functions, they also have many other biological properties, such as antimicrobial activiy.

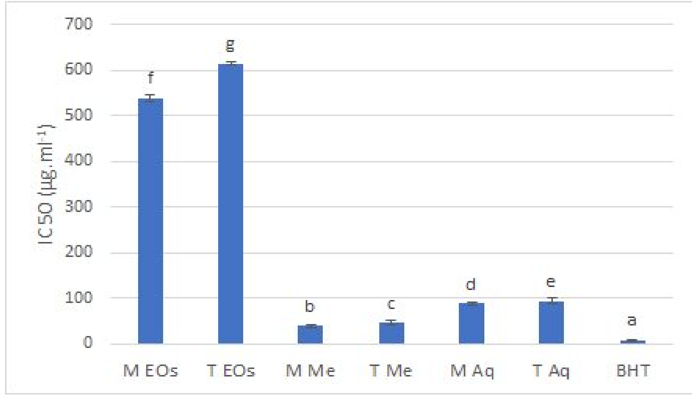


Figure 3. IC_{50} of Eos and extracts of *S.africana.*

M EO: Moroccan essential oil; T EO: Tunisian essential oil; M Me: Moroccan methanolic extract; T Me: Tunisian methanolic extract; M Aq: Moroccan aqueous extract; T Aq: Tunisian aqueous extract.

3.6. Antibacterial Activity

The antibacterial activity of the Eos and crude extracts of *S. africana* aerial parts was evaluated against four bacteria, by the agar disc diffusion method. Results are presented in Table 4 and showed different degrees of bacterial growth inhibition that depended on the strains and the tested sample. Our result showed that essential oils from the two coutries were more effective against *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomona aeruginosa,*with growth inhibitionof 13.33 mm to 15.66 mm. The most important effect was observed against *Staphylococcus aureus*. However, Eos revealed moderate activity against *Salmonella paratyphi* and *Escherchia coli* with inhibition zone around 12mm and 12.5mm, respectively. For methanol and aqueous extracts, a very slight inhibition (9-10.5 mm) was observed against all bacteria strains. As compared to the antibiotics, essential oils from *S. africana* were less effective against the tested bacteria strains, except against *S.aueurs*. Indeed, the same sensitivity of the oils of Morocco and Tunisia (15.33 mm and 15.66 mm, respectively) and ampicillin (16 mm) was observed for this bacteria. In accordance with our results, Malti *et al.*, (2019) showed that Eos of Algerian *S.africana* extracted from the aerial parts at full flowering were effective against *S.aureus* (19.7 mm). Boudjedjou *et al.,* (2019)demonstrated also the potent effect of *S.africana* EO at the fowering stage from Algeria against *E. coli* and *S. aureus* and *B. subtilis,*are respectively, of 29.27 mm, 29 mm and 15 mm.

Liu *et al.*(2007) have also reported that *S.corsica* EO revealed remarkable activity against *S. aureus*, but remaind inactive against *P. aeruginosa* and *E. coli*. However, other studies on *S.chamaecyparissus* and *S.rosmarinifolia* Eos recorded a strong inhibition growth of *E. coli* (25 mm and 15 mm, respectively) (Chibani *et al.*, 2013; Salah-Fatnassi*et al.*, 2017).

S.africana essential oils from Morocco and Tunisia displayed activity against *S.aureus*, *B.subtilis* and *E.coli* with MICs of 7 mg/mL. The tested strains were more resistant against crude extracts. *Ormenis* oils displayed a bacterecidal effect against *S.aure*us and *B.subtilis*, with a MFC of 5 mg/mL (Table 5).

The inherant effects of Eos can be related to their chemical composition, their proportions and to the interactions between their compounds, which may produce additive, synergistic, or antagonistic effects (Tyagi & Malik, 2011). The antibacterial activity of volatile oils can also be attributed to the sensitivity of the tested strains. In fact, essential oils composed mainly of terpenes, phenolsandaldehydes are known by their hydrophobicity that permet them to accumulate in the membranes of bacteria cells and then cause the permeabilityincrease. Leakage of intracellular constituents and alteration of the microbial enzyme can be produced and then cause the loss of cellular content and cell death. In addition, interaction between the different components can occur to changes in the conformation of the structure, causing a reduction in inhibitory activity (Ceylan & Fung, 2004; Bajpai *et al.*, 2013).

Our study revealed that essential oils inhibit more Gram positive bacteria than Gram negative bacteria. This phenomenon was reported previously by Smith-Palmer *et al.,* (1998). It is not known why Gram negative are more resistant, but it can be attributed to the outer membrane that confer to the bacterial surface strong hydrophilicity and acts as strong permeability barrier (Nikaido *et al.*, 1985). Recent data suggest that essential oils may disrupt the permeability barrier of cell membranes and inhibit respiration (Cox *et al.*, 2000).

Table 4. Antibacterial activity of *S.africana* essential oils and extracts.

		Bacteria				
		Gram positive		Gram negative		
		S.aureus	*B.subtilis*	*E.coli*	*P.aeruginosa*	*S. paratyphi*
Eos (10µL/disc)	Morocco	15.33±0.33[a]	14±0.66[c]	12±0.33[b]	13.33±0.57[b]	12[b]
	Tunisia	15.66±0.33[a]	14.66±0.33[b]	12.5±0.33[b]	13.66±0.57[b]	12.33±0.33[b]
MeOH extract (10µL/disc)	Morocco	9[b]	9[d]	-	-	-
	Tunisia	9[b]	9.33±0.33[d]	-	9[c]	9.66±0.33[c]
Aqueous extract (10µL/disc)	Morocco	9[b]	-	-	-	-
	Tunisia	9.33±0.33[b]	-	10[c]	9.66±0.33[c]	-
AMP		16±0.57[a]	-	16.33±0.66[a]	18[a]	15.66±0.33[a]
SPI		-	18.33±0.66[a]	-	-	-
DMSO		-	-	-	-	-

AMP: ampicillin; SPI: spiramycin; DMSO: Diméthylsulfoxide

Table 5. MIC and MBC (mg/mL) of essential oils and extracts from *S.africana.*

		S.aureus		*B.subtilis*		*E.coli*		*P.aeruginosa*		*S. paratyphi*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Eos	Morocco	7	5	7	5	7	-	> 7	-	> 7	-
	Tunisia	7	5	> 7	5	7	-	> 7	-	> 7	-
MeOH extract	Morocco	> 7	-	> 7	-	> 7	-	> 7	-	> 7	-
	Tunisia	> 7	-	> 7	-	> 7	-	> 7	-	> 7	-
Aqueous extract	Morocco	> 7	-	> 7	-	> 7	-	> 7	-	> 7	-
	Tunisia	> 7	-	> 7	-	>7	-	> 7	-	> 7	-

4. CONCLUSION

The present study presents a contribution to the analysis of Eos and extracts from *S. africana* traditionally used for several medicinal applications. The chemical composition of *S. africana* essential oils from Morocco and Tunisia showed their richness in oxygenated monoterpenes. Artemisia ketone and isoborneol were the most abundant in both essential oils. Methanol extracts were rich in phenolic and flavonoid contents and exhibited the highest antiradical activity. Regarding antibacterial property, the evaluation of antibacterial activity using the disc diffusion method and the microdilution method for determining MIC and MBC indicated that *S. aureus* and *B.subtilis* were the most sensitive strains against *S.africana* essential oils.Volatile oils and extracts of *S. africana* can be used as natural agents for the interesting activities they have presented. The chemical composition of each extract may have a crucial role in its bioactivity. However, toxicological investigations are required to demonstrate the safety of this plant.

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

Marwa Khammassi: Investigation, Methodology, Visualization, Formal Analysis, and Writing -original draft, Writing – review & editing. **Sana Khedhri**: Software, Formal Analysis**,** Writing -original draft. **Awatef Slama**: Visualization, Methodology, Formal Analysis**. Meriam Boudkhili:** Data curation, and Formal Analysis, **Ismail Amri:** Investigation, Visualization, Methodology, Supervision, **Lamia Hamrouni**: Supervision, and Validation, **Bassem Jammoussi**: Supervision, and Validation

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REFERENCES

Andrade, M.A., Braga, M.A., Cesar, P.H.S., Trento, M.V.C., Espósito, M.A., Silva, L.F., & Marcussi, S. (2018). Anticancer properties of essential oils: an overview. *Current Cancer Drug Targets*, *18*(10), 957-966. https://doi.org/10.2174/1568009618666180102105843

Bajpai, V.K, Sharma, A., & Baek, K.H. (2013). Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. *Food Control*, *32*(2), 582-590. https://doi.org/10.1016/j.foodcont.2013.01.032

Béjaoui, A., Boulila, A., Ben Salem, I., & Boussaid, M. (2017). Chemical composition and bioactivities of the polyphenolic-rich extract of *Ormenis africana* Jord. and Fourr. *International Journal of Food Properties*, *20*(8), 1786-1795. https://doi.org/10.1080/10942912.2016.1219368

Béjaoui, A., Boulila, A., Mahouachi, I., & Boussaid, M. (2013). Metabolic profile of the bioactive compounds of *Ormenis africana* Jord. and Fourr (Asteraceae) an endemic species from Tunisia. *International Journal of Advanced Research*, *1*(9), 124-131.

Bel Hadj Salah-Fatnassi, K., Hassayoun, F., Cheraif, I., Khan, S., Jannet, H.B., Hammami, M., Aouni, M., & Harzallah-Skhiri, F. (2017). Chemical composition, antibacterial and antifungal activities of flowerhead and root essential oils of *Santolina chamaecyparissus* L., growing wild in Tunisia. *Saudi Journal of Biological Sciences*, *24*(4), 875-882. https://doi.org/10.1016/j.sjbs.2016.03.005

Ben Mansour, R., Gargouri, B., Bouaziz, M., Elloumi, N., Belhadj Jilani, I., Ghrabi, Z., & Lassoued, S. (2011). Antioxidant activity of ethanolic extract of inflorescence of *Ormenis africana* in vitro and in cell cultures. *Lipids in Health and Disease*, *10*(78). https://doi.org/10.1186/1476-511X-10-78

Bouaziz, M., Dhouib, H., Smaoui, S., Loukil, S., Boukhris, M., & Sayadi, S. (2009). Polyphenols, antioxidant and antimicrobial activities of some wild medicinal plants collected in southern Tunisia. *African Journal of Biotechnology*, *8*(24), 7017-7027.

Boudjedjou, L., Ramdani, M., Zeraib, A., Benmeddour, T., & Fercha, A. (2019) .Chemical composition and biological activities of Algerian *Santolina africana* essential oil. *Scientific African*, 4. https://doi.org/10.1016/j.sciaf.2019.e00090

Ceylan, E., Fung, D.Y.C. (2004). Antimicrobial activity of spices. *Journal of Rapid Methods and Automation in Microbiology*, *12*(155). https://doi.org/10.1111/j.1745-4581.2004.tb00046.x

Chang, C.C., Yang, M.H., Wen, H.M., & Chern, J.C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food Drug Analysis*, *10*, 178–182. https://doi.org/10.38212/2224-6614.2748

Chibani, S., Labed, A., Kabouche,A., Semra, Z., Smati, F., Aburjai, T., & Kabouche, Z. (2013) .Antibacterial activity and chemical composition of essential oil of *Santolina rosmarinifolia* L. (Asteraceae) from Algeria. *Der Pharmacia Lettre*, *5*(3), 206–210.

Cox, S.D., Mann, C.M., Markham, J.L, Bell, H.C., Gustafson, J.E., Warmington, J.R., & Wyllie, S.G. (2000). The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology*, *88*(1), 170-175. https://doi.org.10.1046/j.1365-2672.2000.00943.x

Dai, J., & Mumper, R.J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules (Basel, Switzerland)*, *15*, 7313-7352. https://doi.org/10.3390/molecules15107313

Darwish, R.S., Hammoda, H.M., Ghareeb, D. A., Abdelhamid, A.S.A., Bellah El Naggar, E. M., Harraz, F.M., & Shawky, E. (2020). Efficacy-directed discrimination of the essential oils of three *Juniperus* species based on their in-vitro antimicrobial and anti-inflammatory activities. *Journal of Ethnopharmacology*, 259. https://doi.org/10.1016/j.jep.2020.112971

Dewanto, V., Wu, X., Adom, K.K., & Liu, R.H. (2002). Thermal Processing Enhances the Nutritional Value of Tomatoes by Increasing Total Antioxidant Activity. *Journal of Agricultural and Food Chemistry*, *50*(10), 3010-3014. https://doi.org/10.1021/jf0115589

Díaz-Maroto, M.C, Pérez-Coello, M.S, Esteban, J., & Sanz, J. (2006). Comparison of the volatile composition of wild fennel samples (*Foeniculum vulgare* Mill.) from central Spain. *Journal of Agricultural and Food Chemistry, 54*(18), 6814-6818. https ://doi.org/10.1021/jf0609532

Dohoun, N., Yamni, K., Tahrouch, K., Idrissi hassani, L.M, Badoc, A., & Gmira, N. (2003). Screening phytochimique d'une endémique ibéro-marocaine, *Thymelaea lythroides. Bulletin de la Société de Pharmacie Bordeaux*, *142*, 61-78.

European Directorate for the Quality of M. (2008). *Council of European pharmacopoeia*, 6th edn Council of Europe; Stationery Office, Strasbourg, London.

Hatano, T., Edamatsu, R., & Mori, A. (1989) Effects of interaction of tannins with coexisting substances. *Chemical and Pharmaceutical Bulletin*, *38*(8), 2016–2021.

Ismail, A.,Lamia, H., Mohsen, H., Bassem, J., & Kaouthar, L. (2014). Essential oils as biological alternatives to protect date palm (*Phoenix dactylifera* L.) against *Ectomyelois ceratoniae* Zeller (Lepi-doptera: Pyralidae). *Chilean Journal of Agricultural Research*, *74*(3), 273–279. https://doi.org/10.4067/S0718-58392014000300004

Javed, H., Meeran, M.F.N., Jha, N.K., & Ojha, S. (2021). Carvacrol, a Plant Metabolite Targeting Viral Protease (M^{pro}) and ACE2 in HostCells Can Be a Possible Candidate for COVID-19. *Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.601335

Karumi, Y., Onyeyili, PA., & Ogugbuaja, V.O. (2004). Identification of active principles of *M.balsamina* (Balsam Apple) leaf extract. *Journal of Medical Science*, *4*(3), 179-182.

Khammassi, M., Ben Ayed, R., Khedhri, S., Souihi, M., Hanana, M., Amri, I., & Hamrouni, L. (2022). Crude extracts and essential oil of *Platycladus orientalis* (L.) Franco: a source of phenolics with antioxidant and antibacterial potential as assessed through achemometric approach. *Turkish Journal of Agriculture Forestry*, *46*(4), 477-487. https://doi.org/10.55730/1300-011X.3019

Khammassi, M., Ben Ayed, R., Loupasaki, S., Amri, I., Hanana, M., Hamrouni, L., Jamoussi, B., & Khaldi, A. (2023). Chemical diversity of wild fennel essential oils (*Foeniculum vulgare* Mill.): A source of antimicrobial and antioxidant activities. *South African Journal of Botany*, *153*, 136-146. https://doi.org/10.1016/j.sajb.2022.12.022

Khammassi, M., Ben Mansour, M., Laabidi, A., Amri, I., Boudkhili, M., Hamrouni, L., Greeche, H., Khaldi, A., & Jamoussi, B. (2018). Antifungal activity of essential oils and crude extracts of *Santolina africana* from Tunisia and Morocco. *Journal of Material and Environmental Science*, *9*, 2963-2869.

Khammassi, M., Kouki, H., Mighri, H. , Souihi, M., Kochti, O., Seçer, E. , Amri, I., Bassem, J., & Mabrouk, Y. (2023). Phytochemical screening of essential oils and methanol extract constituents of wild *Foeniculum vulgare* Mill.: a Potential natural source for bioactive molecules. *Chemistry Africa*, *6*, 1227-1240. https://doi.org/10.1007/s42250-022-00571-6

Khammassi, M., Mighri, H., Ben Mansour, M., Amri, I., Jamoussi, B., & Khaldi, A. (2022). Metabolite profiling and potential antioxidantactivity of sixteen fennel (*Foeniculum vulgare* Mill.) populations wild-growing in Tunisia. *South African Journal of Botany*, *148*, 407- 414. https://doi.org/10.1016/j.sajb.2022.05.02122

Liu, K., Rossi, P.G., Ferrari, B., Berti, L., Casanova, J., & Tomi, F. (2007). Composition, irregular terpenoids, chemical variability and antibacterial activity of the essential oil from *Santolina corsica* Jordan et Fourr. *Phytochemistry, 68*(12), 1698-1705. https://doi.org/10.1016/j.phytochem.2007.04.027

Lmachraa, I., Fdil, R., Fdil N, & Mouzdahir, A. (2014). Huile essentielle de *Santolina africana* (Jord. & Fourr.) du Maroc:. Composition chimique et isolement des deux principaux constituants (Essential oil of *Santolina africana* (Jord. & Fourr.) of Morocco: Chemical composition and isolation of the two major constituents). *Journal of Material and Environmental Science, 5*, 67-72.

Malti, C.E.W., Baccati, C., Mariani., M., Hassani., F., Babali., B., Atik-Bekkara, F., Paoli, M., Maury, J., Tomi, F., & Bekhechi, C. (2019). Biological activities and chemical composition of *Santolina africana* Jord. et Fourr. aerial part essential oil from Algeria: Occurrence of polyacetylene derivatives. *Molecules, 24*, 204. https://doi.org/10.3390/molecules24010204

Mau, J.L., Chao, G.R., & Wu, K.T. (2001). Antioxydant properties of methanolic extracts from several Ear mushrooms. *Journal of Agricultural and Food Chemistry,* 49, 5461-5467.

Mohammad, R.D., Seyed, A.M.M., Roozbeh, F., & Ali, H. (2022). Changes in seed and shoot essential oil yield of fennel (*Foeniculum vulgare*Mill.) in response to practices of integrated nitrogen management. *Journal of Essential Oil Bearing Plants*, *25*(1), 38-51. https://doi.org/10.1080/0972060X.2022.2026251

N'Guessan, K., Kadja, B., ZirihiGuédé, N., Traoré, D., & Aké-assi, L. (2009). Screening phytochimique de quelques plantes médicinales ivoiriennes utilisées en pays Krobou (Agboville, Côte-d'Ivoire). *Sciences et Nature, 6*(1), 1-15.

National Committee for Clinical Laboratory Standards (NCCLS). (1999). *Performance standards for antimicrobial susceptibility testing*. NCCLS Approved Standard M100-S9. National Committee for Clinical Laboratory Standards, Wayne.

National Committee for Clinical Laboratory Standards (NCCLS). (2003). *Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically, approved standard M7-A6*, 6th ed., Wayne.

Nikaido, H., & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews*, *49*(1), 1-32. https://doi.org/10.1128/mr.49.1.1-32.1985

Perron, N.R., & Brumaghim, J.L. (2009). A Review of the Antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochemistry and Biophysics 53*(2), 75–100. https://doi.org/10.1007/s12013-009-9043-x

Poli, F., Bonsignore, L., Loy, G., Sacchetti, G., & Ballero, M. (1997). *Journal of Ethnopharmacology*, *56*(3), 201-208. https://doi.org/10.1016/s0378-8741(97)01528-6

Pottier Alapetite G. (1981). *Flore de la Tunisie*, 1190.

Quézel, P., & Santa, S. (1963). *Nouvelle flore de l'Algérie et des régions désertiques méridionales*. Éditions du Centre National de la Recherche Scientifique: Paris, France.

Salah-Fatnassi, K.B.H., Hassayoun, F., Cheraif, I., Khan, S., Ben Jannet, H., Hammami M., Aouni, M., & Harzallah-Skhiri, F. (2017). Chemical composition, antibacterial and antifungal activities of flowerhead and root essential oils of *Santolina chamaecyparissus* L., growing wild in Tunisia. *Saudi Journal of Biological Scienc*es, *24*(4), 875-882. https://doi.org/10.1016/j.sjbs.2016.03.005

Saoud, I., Hamrouni, L., Gargouri, S., Amri I ., Hanana, M., Fezzani, T., Bouzid, S., & Jamoussi, B. (2013). Chemical composition, weed killer and antifungal activities of Tunisian thyme (*Thymus capitatus* hoff.et link.) essential oils. *Acta Alimentaria, 42*, 417–427. https://doi.org/10.1556/aalim.42.2013.3.15

Shahidi, F., & Marian, N. (2003). *Phenolics in food and nutraceuticals*. CRS Press LLC, Boca Raton, FL, 1, 144-150.

Slinkard, K., & Singleton, V.L. (1977). Total Phenol Analysis: Automation and Comparison with Manual Methods. *Journal of Enology and Viticulture*, *28, 49-55*. https://doi.org/10.5344/ajev.1977.28.1.49

Smith-Palmer, A., Stewart, J., & Fyfe, L. (1998). Antimicrobial properties of plant essential oil and essences against five important food-borne pathogens. *Letters in Applied Microbiology*, *26*(2), 118-122.

Tundis, R., & Loizzo, M.R. (2018). A Review of the traditional use, phytochemistry and biological activities of the genus *Santolina*. *Planta medica*, *84*(9-10), 627–637. https://doi.org/10.1055/a-0585-6153

Tyagi, A.K., & Malik, A. (2011). Antimicrobial potential and chemical composition of *Eucalyptus globulus* oil in liquid and vapour phase against food spoilage microorganisms. *Food Chemistry, 126*(1), 228–235. https://doi.org/10.1016/j.foodchem.2010.11.002

Wafa, B., Mohsen, H., Samia, G., Ismail, A., Tarek, F., Mustapha, K., Bassem, J., & Lamia, H. (2015). Chemical composition, phytotoxic and antifungal properties of *Ruta chalepensis* L. essential oils. *Natural Product Research*, *29*(9), 864-868. https://doi.org/10.1080/14786419.2014.980246

Wojdyło, A., Oszmiański, J., & Czemerys, R. (2007). Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chemistry*, *105*(3), 940-949. https://doi.org/10.1016/j.foodchem.2007.04.038

Wu, L.C., Hsu, H.W., Chen, Y.C., Chiu, C.C., Lin,Y.I, & Annie Ho, J. (2006). Antioxidant and antiproliferative activities of red pitaya. *Food Chemistry*, *95*(2), 319-327. https://doi.org/10.1016/j.foodchem.2005.01.002

Zaiter, L., Benayache, F., Beghidja, N., Figueredo, G., Chalard, P., Chalchat, J.C, Marchioni, E., & Benayache, S. (2015). Essential oils of *Santolina africana* Jord. & Fourr. and *Santolina chamaecyparissus* L. *Journal of Essential Oil Bearing Plants*, *18*(6), 1338–1342. https://doi.org/10.1080/0972060X.2014.884769

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Research Article

The preliminary study on the antifungal effect of Kaffir lime (*Citrus hystrix* DC) peel extract against *Malassezia furfur*

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Abstract: *Pityriasis versicolor* is one of the most common fungal infections on the skin caused by *Malassezia furfur*. Kaffir lime fruit (*Citrus hystrix* DC), especially its peels' contents in secondary metabolite, may play a healing role against such infections. Therefore, the aim of this study was to determine the antifungal potential of Kaffir lime peel extract against *Malassezia furfur*. Ethanol extracts of the peels of Kaffir lime were tested for its antifungal properties against *Malassezia furfur* at different tested concentrations, namely 25%, 50%, 75%, and 100%. Every treatment was performed with 4 replications. Inhibition zone formed surrounding the paper disc measured after 24 hours of incubation using the caliper method. Data were subjected to the Kruskal-Wallis test and the Mann-Whitney Post hoc test. The study revealed that the kaffir lime fruit extract gave a significant effect on the inhibition zone ($p < 0.05$). The results showed that the higher inhibition zone was found at control group and it was different significantly from other treatments. However, among the tested concentrations, the best treatment was detected at a concentration of 100%, which was significantly different from other concentrations. Kaffir lime peel has an antifungal effect against *Malassezia furfur* and the best tested concentration was 100%.

1. INTRODUCTION

Skin infection is a common disease in tropical countries, including Indonesia, where fungi are the most frequent pathogens that cause such diseases. Fungal skin diseases were the most common skin diseases worldwide in 2017 (10.09%). In addition, skin fungus was the fourth most common disease (2.1 billion cases) compared to 328 diseases and injuries worldwide (Alastruey-Izquierdo *et al*., 2015; Celis *et al*., 2017; Ariana, 2018). *Malassezia* is a lipophilic fungus which commonly finds normal flora on human skin. *Malassezia furfur* is one of the species of fungus that causes *pityriasis versicolor*. *Malassezia furfur* is a yeast that leads to skin lesions when it transforms into a pathogenic filament (Georgios Gaitanis *et al*., 2012; Agusrimansyah *et al*., 2019).

Currently, the treatment for *P. versicolor* is by administering topical drugs as the first line therapy and systemic antifungal drugs as the second line. Ketoconazole is one of the common

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topical treatments in cream or shampoo preparations for this infection. However, antifungal drugs have some limitations, such as severe side effects, narrow spectrum, minimal penetration of some tissues, and potential of resistance (Gupta & Foley, 2015; Pusung *et al*., 2021; *Lee et al*., 2023). The resistance of anti-fungal drugs has been reported by several researchers; for instance, the incidence of *Candida albicans* resistance to the antifungal fluconazole was 34.07%, and resistance to ketoconazole was 7.69% (Apsari & Adiguna, 2013). Helou *et al* (2014) reported that there was a case related to a 52-year-old man who had *P. versicolor* and developed resistance to fluconazole, ketoconazole, itraconazole, and fenticonazole cream as a result of repeated cases and ongoing usage of these medications without doctor's advice (Helou *et al*., 2014).

Thus, it is essential to investigate various alternative materials as antifungal agents to avoid the resistance case. The contents of secondary metabolite from plants may potentially be used as substances in phytotherapeutic approaches. According to Masloman *et al* (2016), the active materials compound from plants has low side effects, does not cause resistance, and is easy to obtain and relatively inexpensive (Masloman *et al*., 2016). Kaffir lime (*Citrus hystrix* DC), especially the peel of the fruit, has active metabolites compound such as saponins, tannins, flavonoids and steroids which have potential as antifungals (Rachmatiah & Octaviani, 2022).

The previous report by Halawa *et al* (2019) shows that kaffir lime peel extract has a potential inhibition effect on the growth of *Aspergillus niger* and *Candida albicans* (Halawa *et al*., 2019). Another study also reported that kaffir lime leaf extract with concentrations of 25%, 50% and 75% could inhibit the growth of *Pityrosporum oval* (Ramadhani *et al*., 2021). In addition, Khafidhoh *et al.* (2015) reported that the kaffir lime peel infusion with concentrations of 10%, 15% and 20% gave an antifungal effect on the growth of *Candida albicans* in vitro (Khafidhoh *et al*., 2015). To date, there has been no study on the potency of kaffir lime peel as antifungal for *Malassezia furfur*. Therefore, the objective of this study was to investigate the antifungal activity of Kaffir lime peel extract against *M. furfur* and established the optimal concentration.

2. MATERIAL and METHODS

2.1. Experimental Design

The research, a non-factorial experiment, used a completely randomized design. The treatment was based on differences in kaffir lime peel extract at four tested concentration, namely 25%, 50%, 75% and 100%. The sample of *M. furfur* ATCC. 14521 consists of five groups, namely control group, group 1 for 25% concentration, group 2 for 50% concentration, group 3 for 75% concentration, and group 4 for 100% concentration of the kaffir lime peel extracts. The diameter of the *M.furfur* growth inhibition zone was the assessed variable.

2.2. *Malassezia furfur* Colonies Preparation

The *Malassezia furfur* fungal was cultured using sterile ose and strake on Sabouroud Dextrose Agar (SDA) + olive oil medium which had solidified (Purwanti & Susanti, 2016). Then, the media were incubated at 37^oC for 18-24 h until *M. furfur* colony growth (Silvia *et al*., 2015). *Malassezia furfur* colonies were then separated into five test groups. Ketoconazole was used as a control. The solution ketoconazole was made by mixing 200 mg ketoconazole tablets which were crushed and diluted in 10 mL of sterile distilled water (Maulana *et al*., 2020). The treatment group was given extracts with respective tested concentrations (25%, 50%, 75% and 100%). A previous study reported that the antifungal effect of kaffir lime peel extract had the Minimum Inhibitory Concentration (MIC) at a concentration of 25% (Halawa *et al*., 2019).

2.3. Kaffir Lime Peels Extract Preparation

Kaffir lime extracts were prepared by taking peels parts, macerated in ethanol 96%, and then stored for 5 days. The extraction solution was filtered using a filter paper and a glass funnel. The filtrated biomass was collected in a sealed beaker glass. The maceration process was repeated using the medicinal residue once by using the same extraction solvent. The two

filtrates were pooled together and then drained using a rotary evaporator at 60°C with a pressure of 60 rpm for 8 hours (Halawa *et al*., 2019). Phytochemical test on Kaffir lime peels extracts was examined to define secondary metabolites including saponins, tannins, alkaloids, flavonoids, steroid, and terpenoid. The qualitative test identifies the presence of certain secondary metabolites by a color reaction or precipitate as a positive result using Mayer's reagent, Libermann-Burchard reagent, and FeCl 5% (Julianto, 2019).

2.4. Antifungal Activity Test

The difference in extract concentration can be prepared by diluting the kaffir lime peel extract with DMSO**.** 25% extract concentration can be prepared by diluting 2.5 ml of kaffir lime peels extract with 7,5 ml of DMSO, 50% concentration by diluting 50 ml of kaffir lime peel extract, and 50 ml of DMSO, and 75% concentration (4:1) with 7,5 ml of kaffir lime peel extract and 2,5 ml of DMSO. A positive control can be prepared by diluting 200 mg ketokonazol with 10 ml sterile distilled water. A negative control uses DMSO 100% (Maulana *et al*., 2020). In our study a blank disc paper with a 6 mm diameter was tested for antifungal activity. One milliliter of extract from kaffir lime peels was added to the disc sheets. The plates were allowed to incubate at 32°C during 48 hours. The presence of a clear zone indicated that fungus growth was inhibited, and the diameter was calculated in millimeters using a caliper. The inhibition zone was determined based on the diameter of the filter paper disc minus the diameter of the disc paper employed (Mulyadi *et al*., 2017). The inhibitory response of fungal growth according was determined according to Davis and Stout (1971), namely diameters: >20 mm, very strong; 10-20 mm, strong, 5-10 mm, medium; and <5 mm, no response.

2.5. Data Analysis

The data were subjected to Kruskal-Wallis test to examine the effect of kaffir lime peels extract on the growth of *M. furfur* at different concentrations. A Mann- Whitney Post hoc test was conducted to identify the optimal concentration of kaffir lime peel extract on the *M. furfur* growth inhibition.

3. RESULTS

3.1. Antifungal Effect of Kaffir Lime

The antifungal activity test showed that the inhibition zone was formed at the control group, and all tested concentrations. Among the tested concentrations, a concentration of 25% formed the smallest diameter of inhibition (9.7 mm), while the largest inhibition diameter was formed at a concentration of 100% (24.5 mm). Figure 1 shows the inhibition diameter formed at respective tested concentrations. The zone of inhibition was a circular area around the discs of the Ketoconazole and kaffir lime peels extract in which the *M. furfur* colonies did not grow.

Our study revealed that the inhibition zone at a concentration of 25% had a moderate category and had strong category at a concentration of 50% and 75% while it had strongest category at a concentration of 100%. Kruskal-Wallis test revealed that concentration of kaffir lime peel extracts gave a significant effect on the inhibition zone of *M. furfur* ($p< 0.05$). The higher inhibition zone was found at a concentration of 100% (24.5 mm). This value was significantly different for all groups except the control group according to the Mann Whitney post hoc test. Therefore, the concentration of 100% of kaffir lime peels extract showed the most effective antifungal effect against *M. furfur* among the other tested concentrations (Table 1).

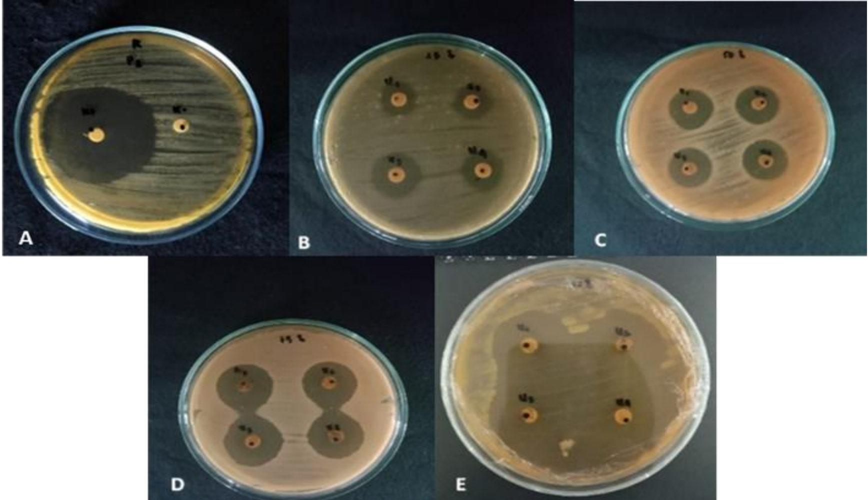

Figure 1. Streaking results of Ketoconazole (A); Kaffir lime peel extract on Group 1 (25%) (B), Group 2 (50%) (C), Group 3 (75%) (D), Group 4 (100%) (E).

Tabel 1. The inhibition zone of the growth *Malassezia furfur* between groups.

Parameters	Control	Group 1 (25%)	Group 2 (50%)	Group 3 (75%)	Group 4 (100%)
Inhibition zone	46.5±4.0[a]	9.7±1.7[e]	14.1±0.8[d]	18.2±0.3[c]	24.5±3.3[b]

Notes: Values are mean zone of inhibition diameter ± Standard Deviation. Different superscripts within the same row mean statistical difference at ($p < 0.05$).

3.2. Result of Identification Secondary Metabolite

Based on phytochemical essay kaffir lime peels extract contained secondary metabolites such as alkaloids, saponins, tannins, flavonoids, steroids, and terpenoid as observed in this study (Table 2).

Table 2. The phytochemical essay of secondary metabolites**.**

Secondary metabolites	Reagent	Result
Alkaloid	Bouchardart	+
	Maeyer	+
Saponin	Aquadest + Alkohol 96% +HCl 2N	+
Tannin	$FeCl_3$ 5%	+
Flavonoid	$FeCl_3$ 5%	+
Steroid	Salkowsky	+
	Liebermann-Bouchard	+
Terpenoid	Salkowsky	+
	Liebermann-Bouchard	+

The positive results of alkaloid test in Wagner test were confirmed by the presence of brownish to yellowish precipitate as Bouchardart test identified brownish precipitate as well. The foam formation in sapponin test provided the presence of glycosides that have an ability to produce foam in water hydrolyzed in glucose and other compounds. The positive results of alkaloid test in $FeCl_3$ 5% test were confirmed by the presence of blackish green precipitate. The extract samples that contained flavonoid compounds form the black flavium salt after addition of FeCl3 5%. Steroid/terpenoid screening is based on Salkowsky test, a positive result with a marked reddish color change indicating triterpenoid content and golden yellow steroid. The addition of Liebermann-Bouchard was confirmed by the presence of brown ring indicating triterpenoid content and greenish blue color change for steroid.

4. DISCUSSION

This study has shown that Kaffir lime peels extract at all tested concentrations inhibits the growth of the *M. furfur* in vitro. The formation of an inhibition zone indicated by the clear zone around the disc resulted in the moderate category at a concentration of 25%, and at 50% and 75% concentrations gave strong category, while at 100% concentration resulted in a very strong category. Thus, the results of this study showed the kaffir lime peels extract had potential as an antifungal effect. The study revealed that the diameter inhibitor zone increased proportionally with the increasing Kaffir lime extract concentration. The study conducted by Choirunnisa *et al.* (2022) reported that the ability of an antimicrobial depends on the concentration and the type of antimicrobial produced (Choirunnisa *et al*., 2022). The diameter of the inhibition zone increases with increasing extract concentration as recorded in this study. This is due to a higher concentration, ingredients of antimicrobials are also increasing. Besides inhibit the M. furfur, the kaffir lime peel extract also inhibited the growth of Staphylococcus aureus where the inhibition zone was increased as increases of kaffir lime peel extracts concentration (Madduluri *et al*., 2013).

The results of phytochemical essay kaffir lime peel extracts contained secondary metabolites as essentials materials that play antimicrobials and antifungal activities. For instance, flavonoids inhibit the growth of fungi by damaging the permeability of the plasma membrane, disrupt the mitochondria, disrupt the fungal cell wall synthesis, and inhibit RNA and protein synthesis resulting in disrupted reproduction and metabolism fungi (Serpa *et al*., 2012; Sari DP *et al*., 2017; Al Aboody & Mickymaray, 2020; Susilawati *et al*., 2023). Alkaloids have antifungal activities that result in disruption of the cell respiration system and protein synthesis, can trigger the loss of nutrients in fungal cells, slow reproduction of fungi, and even cell death *(Dhamgaye et al*., 2014; Swandiyasa *et al*., 2019). Alkaloids as an antifungal can destroy peptidoglycan in the fungal cell wall and trigger disruption of the permeability of the fungal cell wall. The decrease in the proliferation of fungal is due to the presence of saponin activity that damages the permeability of the fungal membrane that results in the leakage of cellular materials including nucleic acids and proteins (Lely & Rahmanisah, 2017; Porsche *et al*., 2018; *Dong et al.*, 2020).

Tannin can inhibit the ergosterol formed as the protein structure and regulatory components of the fungal cell membrane. Steroids can destroy the lipid membranes, which leads to the leakage of liposomes (Ernawati & Sari, 2015; Arifin *et al*., 2018). Additionally, it is known that steroids can penetrate lipophilic substances to interact with phospholipid membranes and thus can impair the cell membrane's integrity, resulting in cell lysis (Madduluri *et al*., 2013; Anggraini *et al*., 2019; Subaryanti *et al*., 2022). Terpenoids have the potential to interact with porins, which are transmembrane proteins in the outer membrane of the fungal cell wall. Terpenoids have the potential to interact with porins, which are transmembrane proteins in the outer membrane of the fungal cell wall. Terpenoids and porin create polymer bonds that can destroy the protein structure, lead to decrease in permeability, and therefore trigger bacterial cells to lose nutrients and even die (Ernawati & Sari, 2015; Amalia *et al*., 2017). There are several uses of kaffir lime peels, namely it has potential as a herbal shampoo for dandruff treatment because it is effective against dandruff microbial and also kaffir lime peel can potentially be a disinfectant solution on cutlery to reduce germ numbers (Tanzil *et al*., 2017; Rusmiati *et al*., 2023).

5. CONCLUSION

In conclusion, this study shows that the Kaffir lime peels extract has a potential antifungal activity against M. furfur while its concentration of 100% is the best concentration to inhibit the growth of this fungal, possibly due to its secondary metabolite content. The findings obtained in the current study therefore lead us to believe that Kaffir lime peels extract has potential as antifungal against M. furfur.

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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). This research was approved by the Research Ethics Committee Malikussaleh University Faculty of Medicine. **Ethics Committee Number**: 10/KEPK/FKUNIMAL-RSUCM/2023.

Authorship Contribution Statement

Mulyati Sri Rahayu and **Wizar Putri Mellaratna:** Investigation, Methodology, Validation Resources, Formal Analysis, and Writing - original draft. **Nailah Najah:** Investigation, Visualization, Software, and Writing.

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REFERENCES

Agusrimansyah, K., Soleha, T.U., Gita Mutiara, U., & Juli. (2019). Perbandingan Efektivitas Lidah Buaya (*Aloe vera*) dan Buah Adas (*Foeniculum vulgare*) Terhadap Pertumbuhan Malassezia furfur. *Repository. Lppm. Unila. Ac. Id*, *9*(1), 71.

Al Aboody, S.M., & Mickymaray, S. (2020). Antibiotics Anti-Fungal Efficacy and Mechanisms of Flavonoids. *Antibiotics*, *9*(45), 25–26.

Alastruey-Izquierdo A, M.M., LX, B., & JL, R.-T. (2015). Susceptibility test for fungi: clinical and laboratorial correlations in medical mycology. *Revista Do Instituto de Medicina Tropical de São Paulo, 57*(19), 60.

Amalia, A., Sari, I., & Nursanty, R. (2017). Aktivitas Antibakteri Ekstrak Etil Asetat Daun Sembung (*Blumea balsamifera* (L.) DC.) terhadap Pertumbuhan Bakteri Methicillin Resistant Staphylococcus aureus (MRSA). *Jurnal UIN Ar-Raniry*, *5*(1), 390.

Anggraini, W., Nisa, S.C., Da, R.R., & Ma, B. (2019). Aktivitas antibakteri ekstrak etanol 96 % buah blewah (*Cucumis melo* L . var . *cantalupensis*) Antibacterial activity of 96 % ethanol extract cantaloupe fruit (*Cucumis melo* L . var . *cantalupensis*) against *Escherichia coli* bacteria. *Pharmaceutical Journal of Indonesia*, *5*(1), 61–66.

Apsari, A.S., & Adiguna, S. (2013). Resistensi Antijamur dan Strategi Untuk Mengatasi. *Journal of Media Dermato-Venerologica Indonesia*, *40*(2), 89–95.

Ariana, D. (2018). Perbedaan Zona Hambat Terhadap Jamur Malassezia Furfur Antara Pemberian Ekstrak Umbi Bawang Putih (*Allium sativum* Linn) Dengan Ekstrak Kulit Umbi Bawang Putih (*Allium sativum* Linn). *The Journal of Muhammadiyah Medical Laboratory Technologist*, *1*(2), 166–167. https://doi.org/10.30651/jmlt.v1i2.1501

Arifin, Z., Khotimah, S., & Rahmayanti, S. (2018). Aktivitas Antijamur Ekstrak Etil Asetat Daun Mangga Bacang (*Mangifera foetida* L .) terhadap *Candida albicans* secara In Vitro. *Jurnal Cerebellum*, *4*(3), 1114.

Celis, A.M., Vos, A.M., Triana, S., Medina, C.A., Escobar, N., Restrepo, S., Wösten, H.A.B., & de Cock, H. (2017). Highly efficient transformation system for Malassezia furfur and Malassezia pachydermatis using *Agrobacterium tumefaciens*-mediated transformation. *Journal of Microbiological Methods*, *134*, 1–6. https://doi.org/10.1016/j.mimet.2017.01.001

Choirunnisa, G.N., Kurniati, I.D., & Ratnaningrum, K. (2022). Antimicrobial potential of Kaffir Lime (*Citrus hystrix* D.C) peel extract against *Staphylococcus aureus*. *Jurnal*

Saintika Medika, *18*(1), 18–27.

Dhamgaye, S., Devaux, F., Vandeputte, P., Khandelwal, N.K., Sanglard, D., Mukhopadhyay, G., & Prasad, R. (2014). Molecular mechanisms of action of herbal antifungal alkaloid berberine, in *Candida albicans. PLoS One*, *9*(8), e104554. https://doi.org/10.1371/journal.pone.0104554

Dong, S., Yang, X., Zhao, L., Fengxiang Zhang, Z.H., & Xue, P. (2020). Antibacterial activity and mechanism of action saponins from *Chenopodium quinoa* Willd. husks against foodborne pathogenic bacteria, Industrial Crops and Products. *Industrial Crops and Product*, *149, July 2020, 112350*. https://doi.org/https://doi.org/10.1016/j.indcrop.2020.112350

Ernawati, & Sari, K. (2015). Kandungan Senywa Kimia dan Aktivitas Antibakteri Ekstrak Kulit Buah Alpukat (*Persea americana* P.Mill) Terhadap Bakteri Vibrio alginolyticus. *Jurnal Kajian Veteriner*, *3*(2), 207–208. https://doi.org/10.35508/jkv.v3i2.1043

Georgios Gaitanis, P.M., Hantschke, M., Bassukas, I.D., & Velegraki, A. (2012). The Malassezia genus in skin and systemic diseases. *Clinical Microbiology Reviews*, *25*(1). https://doi.org/10.1128/cmr.00021-11

Gupta, A.K., & Foley, K.A. (2015). Antifungal treatment for pityriasis versicolor. *Journal of Fungi*, *1*(1), 14. https://doi.org/10.3390/jof1010013

Halawa, C.W.D., Mendrofa, E., & Lubis, Y. (2019). Uji Efektivitas Antijamur Ekstrak Kulit Jeruk Purut (Citrus Hystrix) Terhadap Pertumbuhan Jamur *Aspergillus niger* Dan Candida Albicans. *Jurnal Biosains*, *5*(1), 39–41. https://doi.org/10.24114/jbio.v5i1.12313

Helou, J., Obeid, G., Moutran, R., & Maatouk, I. (2014). Pityriasis versicolor: a case of resistance to treatment. *International Journal of Dermatology*, *53*(2), 114.

Julianto, T.S. (2019). *Fitokimia Tinjauan Metabolit Sekunder dan Skrining Fitokimia*. Yogyakarta: Universitas Islam Indonesia. https://chemistry.uii.ac.id/Tatang/Fitokimia.pdf

Khafidhoh, Z., Dewi, S.S., & Iswara, A. (2015). Efektivitas Infusa Kulit Jeruk Purut (*Citrus hystrix* DC.) Terhadap Pertumbuhan *Candida albicans* Penyebab Sariawan Secara in vitro. *Jurnal Medika Veterinaria*, *7*(2), 31–32. https://doi.org/10.21157/j.med.vet..v7i2.2951

Lee, S.-H., Jang, Y.-B., Choi, Y., Lee, Y., Shin, B.N., Lee, H.-S., Lee, J.-S., & Bahn, Y.-S. (2023). Adenylyl-Sulfate Kinase (Met14)-Dependent Cysteine and Methionine Biosynthesis Pathways Contribute Distinctively to Pathobiological Processes in *Cryptococcus neoformans*. *Microbiology Spectrum*, *11*, e00685-23. https://doi.org/10.1128/spectrum.00685-23

Lely, N., & Rahmanisah, D. (2017). Uji Daya Hambat Minyak Atsiri Rimpang Kencur (Kaempferia galanga Linn) Terhadap *Trichophyton mentagrophytes, Trichophyton rubrum. Jurnal Penelitian Sains*, *19*(2), 94–99.

Madduluri, S., Babu Rao, K., & Sitaram, B. (2013). In vitro evaluation of antibacterial activity of five indigenous plants extract against five bacterial pathogens of human. *International Journal of Pharmacy and Pharmaceutical Sciences*, *5*(4), 683.

Masloman, A.P., Pangemanan, D.H. ., & Anindita, P. (2016). Uji Daya Hambat Ekstrak Daun Sirsak (*Annona murcata* L.) terhadap Pertumbuhan Jamur *Candida albicans. Jurnal Ilmiah Farmasi*, *5*(4), 61–68.

Maulana, R., Zulfa, F., & Setyaningsih, Y. (2020). Uji efektivitas ekstrak kulit pisang ambon (*Musa paradisiaca* var *sapientum* L.) terhadap pertumbuhan *Trichophyton rubrum* secara in vitro. *Seminar Nasional Riset Kedokteran*, *1*(1), 1-7. https://conference.upnvj.ac.id/index.php/sensorik/article/view/426

Mulyadi, M., Wuryanti, W., & Sarjono, P.R. (2017). Konsentrasi Hambat Minimum (KHM) Kadar Sampel Alang-Alang (Imperata cylindrica) dalam Etanol Melalui Metode Difusi Cakram. *Jurnal Kimia Sains Dan Aplikasi*, *20*(3), 130-135. https://doi.org/10.14710/jksa.20.3.130-135

Porsche, F.M., Molitor, D., Beyer, M., Charton, S., André, C., & Kollar, A. (2018). Antifungal activity of saponins from the fruit pericarp of sapindus mukorossi against *Venturia inaequalis* and *Botrytis cinerea*. *Plant Disease*, *102*(5), 991-1000. https://doi.org/10.1094/P

DIS-06-17-0906-RE

Purwanti, N.U., & Susanti, R. (2016). Uji Aktivitas Antibakteri dan Antifungal Ekstrak Etanol Rimpang Acorus sp. *Jurnal Kesehatan Khatulistiwa*, *2*(1), 262.

Pusung, A.V., Suling, P.L., & Niode, N.J. (2021). Efektivitas Pengobatan Topikal pada Pitiriasis Versikolor. *E-CliniC*, *9*(1), 144. https://doi.org/10.35790/ecl.v9i1.32119

Rachmatiah, T., & Octaviani, R. (2022). Aktivitas Antifungi Ekstrak Daun Bisbul (Diospyros blancoi A. DC.) terhadap *Trichophyton mentagrophytes* dan Malassezia furfur. *Sainstech Farma: Jurnal Ilmu Kefarmasian*, *15*(2), 57-64. https://doi.org/https://doi.org/10.37277/sfj.v15i2.1288

Ramadhani, S.L.N., Napiah, A.N., & Wahyuni, S.N. (2021). An Experiment for Extracted Citrus Hystrix Leaf Effectiveness on Pityrosporum Ovale Fungi Growth. *Himbep, 2020*, 294–295. https://doi.org/10.5220/0010352902910295

Rusmiati, R., Suwanta, D.N.P., Ipmawati, P.A., & Marlik, M. (2023). The Use of Kaffir Lime Peel Filtrate (*Citrus hystrix*) in Reducing The Number of Cutlery Germs. *Jurnal Kesehatan Lingkungan Indonesia*, *22*(1), 55–59. https://doi.org/10.14710/jkli.22.1.55-59

Sari, D.P., Nahzi, M.Y.I., & Budiarti, L.Y. (2017). Efektivitas Daya Hambat Ekstrak Umbi Bawang Dayak Terstandarisasi Fenol Terhadap Pertumbuhan *Enterococcus faecalis*. *Dentino Jurnal Kedokteran Gigi*, *1*(1), 56–61.

Serpa, R., França, E.J.G., Furlaneto-Maia, L., Andrade, C.G.T.J., Diniz, A., & Furlaneto, M.C. (2012). In vitro antifungal activity of the flavonoid baicalein against *Candida* species. *Journal of Medical Microbiology*, *61*(12), 1704-1708. https://doi.org/10.1099/jmm.0.047852-0

Silvia, Savante, A., & Muhamad, A.W. (2015). Aktivitas Antimikroba Ekstrak Daun Soma (*Ploiarium alternifolium* Melch) Terhadap Jamur Malassezia furfur dan Bakteri *Staphylococcus aureus*. *Jurnal Kimia Khatulistiwa*, *4*(3), 84-93.

Subaryanti, S., Melasari, F., & Zainuddin, R. (2022). Potensi Antifungi Ekstrak Etanol Kulit Buah Pisang Batu (*Musa balbisiana* Colla) Terhadap Pertumbuhan *Candida albicans* dan *Candida tropicalis*. *Sainstech Farma*, *15*(1), 23–30. https://doi.org/10.37277/sfj.v15i1.1107

Susilawati, S., Anwar, C., Saleh, I., & Salni, S. (2023). Flavonoid as Anti-Candida Agents. *Indonesian Journal of Fundamental and Applied Chemistry*, *8*(2), 88-97. https://doi.org/10.24845/ijfac.v8.i2.88

Swandiyasa, K., Puspawati, N.M., & Asih, I.A.R.A. (2019). Potensi Ekstrak Daun Cendana (*Santalum album* L.) Sebagai Senyawa Penghambat Jamur *Candida albicans*. *Jurnal Kimia*, *13*(2), 164. https://doi.org/10.24843/jchem.2019.v13.i02.p06

Tanzil, L., Latirah, L., & Nugroho, P.D. (2017). Antidandruff Activityof Extracts From Kaffir Lime (*Citrus hystrix* Dc.) Prepared By Different Solvents. *Sanitas: Jurnal Teknologi Dan Seni Kesehatan, 8*(1), 57-62. https://doi.org/10.36525/sanitas.2017.9

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journal homepage: https://dergipark.org.tr/en/pub/ijsm **Research Article**

Antibacterial and antioxidant properties and phytochemical screening of *Laurus nobilis* L. extract from Ethiopia

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Abstract: Microbial resistance to antibiotics and the shortage of efficient antimicrobial agent has necessitated the search for a better antimicrobial agent from various sources. Plants secondary metabolites are the major sources for discovery of new bioactive chemical compounds. The objective of this study was to determine the antibacterial and antioxidant activities of *Laurus nobilis* leaf extract and its essential oil against human pathogenic microorganisms and to analyse its chemical composition. The leaf of *L. nobilis* (500 g) was air-dried, powdered and extracted using four different solvents. The crude extract and the essential oil were tested against four Gram-negative and two Gram-positive bacterial strains. The radical scavenging activity of the crud extract was examined using DPPH assay. Bacterial inhibition activity of the crude extract increased with increased concentration from 25 mg/mL to 200 mg/mL. The maximum inhibition zone was recorded against *Enterococcus faecalis* 13.33±1.52 mm, *Escherichia coli* 14.33±1.53 mm and *Salmonella typhimurium* 16.00±1.00 mm, respectively. MeOH extract (1000 µg/mL) showed superior radical scavenging property (0.02) than ascorbic acid (0.05). The analysis of the oil using GC-MS indicated the presence of 48 chemical substances accounting for 91.4 % of the total compositions. The finding of this study showed that bay leaf has considerable antimicrobial and antioxidant activities. Further evaluation of this plant is recommended with particular focus on the mechanisms of action of the antimicrobial substance.

1. INTRODUCTION

Bay leaf (*Laurus nobilis* L.) is a perennial aromatic evergreen tree or large shrub with smooth leaves classified in the laurel family (Lauraceae). The plant is an important component in culinary and many traditional practices (Parthasarathy *et al*., 2008). The antimicrobial activity of this plant has been reported against several infections including fungi, viruses, bacteria, and protozoa (Fukuyama *et al*., 2011). *L. nobilis* has been used as herbal preparation to increase perspiration for diseases like rheumatism, sprains, dyspepsia, and earaches (Fang *et al*., 2005). The juice of this plant is an efficient treatment for sore eyes and night blindness resulted from vitamin A deficiency. The seeds of bay plant are also reported to have relieved indigestion, sore

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throat, constipation, and diarrhoea (Batool *et al*., 2019). Additionally, the cosmetic industry employs its essential oil in creams, perfumes, and soaps (Sharmeen *et al*., 2021).

In Ethiopia, there is no sufficient information on the antibacterial and antioxidant activities and phytochemical analysis of *L. nobilis.* Thus, the objective of this work was to investigate the antibacterial and antioxidant activities of *L. nobilis* leaf extract against selected human pathogenic microorganisms.

2. MATERIAL and METHODS

2.1. Plant Material

The *L. nobilis* leaf was collected in January 2021 from Addis Ababa, Ethiopia, and brought to Adama Science and Technology University. Voucher sample of the plant was prepared and pressed on a newspaper separately for taxonomic identification. The taxonomy of the plant was determined at the Department of Plant Biology and Biodiversity Management, Addis Ababa University.

2.2. Preparation of Plant Extract

In this study, the solid-liquid extraction technique was employed as a general extraction method. Total extraction of plant material was made by mixing the dried and grounded *L. nobilis* sample with organic solvents. Air-dried and powdered plant material (0.5 kg) was extracted with methanol (2.5 L), macerated at ambient temperature by placing it on a shaker for 24 h and filtered with Whatman No. 1 filter paper. Then, using rotary evaporator, the filtrate was concentrated and dried. The dried methanol extract was suspended and consecutively partitioned twice with equal volumes of n-hexane, chloroform ($CHCl_3$), and ethyl acetate (EtOAc). All solvent fractions were filtered and the filtrates were dried at 40 ^oC using rotavap and maintained in a vial in a refrigerator until further use.

2.3. Phytochemical Screening

The preliminary screening of phytochemical constituents of crude extract of the *L. nobilis* was done to investigate compounds including alkaloids, flavonoids, saponins, tannins, phenols, terpenoids, and steroids using the standard procedures.

2.3.1. *Test for steroid*

Two mL of acetic anhydride was mixed with *L. nobilis* extracts previously dissolved in 2 mL of sulphuric acid. The change of appearance from violet to green confirmed the existence of steroids.

2.3.2. *Test for terpenoid*

Five mL of the extract and 2 mL chloroform were mixed and then combined with with 3 mL concentrated H_2SO_4 (Salkowski test). The formation of reddish-brown color at the interface indicated the existence of terpenoids.

2.3.3. *Test for saponins*

Two to three drops of distilled water were mixed with one millilitre of the crud extract and the mixture was violently agitated. The presence of saponins was determined by persistent foaming.

2.3.4. *Test for flavonoids*

Three mL of *L. nobilis* crude extract and fractions were treated with a few drops of sodium hydroxide solution. The formation of an intense yellow color, which becomes colorless with the addition of dilute acid indicates the presence of flavonoids.

2.3.5. *Test for tannins*

The leaf extract (about 0.25 g) of was heated in 20 mL of water in a test tube. Then, the solution was filtered and mixed with 2-3 drops of 0.1% Iron III chloride. The existence of tannins was confirmed by the observation of a green-blackish color.

2.3.6. *Alkaloids test*

One mL hydrochloric acid (1%) was added to 3 mL of the leaf extract. The solution was boiled for 20 minutes and filtered after cooling. Then one millilitres of the filtrate was mixed with 0.5 mL of Mayer's reagent. The appearance of a yellow precipitate reveals the existence of alkaloids.

2.3.7. *Test for phenol*

The solution prepared from the leaf extract (2-3 drops) was mixed with a few drops of ferric chloride reagent. The appearance of a bluish-black color indicates the existence of phenols.

2.4. Extraction of essential oils (EOs)

L. nobilis leaf (60 g) was powdered by an electrical grinder and extracted for 2 h by hydro-distillation method using 500 mL distilled water in a Clevenger apparatus. The EOs were taken and dried using anhydrous Na_2SO_4. The yield of the oil was determined as follows and kept in sealed vials at 4 °C in a refrigerator until further analysis.

$$Yieald\ /\%) = \frac{Amount\ of\ extracted\ oil\ (g)}{Amount\ of\ dry\ plant\ material} x100$$

2.5. GC-MS Analysis

Investigation of the EOs was done by GC-MS; GC (7890B, Agilent Technologies) coupled with an MS (5977A Network). The GC had an HP-5MS column (30 µm × 250 µm (i.d.) and 0.25 µm). The GC-MS method of Hanus *et al*., 2008 was used for the analysis. Helium 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 10 ºC/min (inlet 250 °C; detector 280 °C; split less injection/purge time 1.0min), 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. Identification of the components in the oils carried out by comparing the mass spectra of the samples with the database of NIST11 GC-MS libraries.

2.6. Antibacterial Assay

Antibacterial assay of *L. nobilis* leaf extract and essential oil was performed by disk diffusion method in Mueller Hinton Agar (MHA) plates. Four different concentrations were prepared from the extract (200, 100, 50, and 25 mg/mL) and the oil (45, 22. 5, 11.25, and 5.6 µg/mL) using DMSO as a solvent. Bacterial cultures of *S. aureus* (ATCC25923), *E. faecalis* (ATCC29212), *P. aeruginosa* (ATCC27853), *E. coli* (ATCC25922), *S. typhimurium* (ATCC13311) and *K. pneumonia* (ATCC700603),obtained from Adama Public Health Research and Referral Laboratory (Ethiopia) were grown in blood agar media at 37 °C for 24 h. The colonies were adjusted to 1.5 $\times 10^8$ CFU/mL (Andrews, 2002) using 0.5 McFarland standard (Saeed & Tariq, 2007) and maintained in a flask to compare the bacterial turbidity.

2.6.1. *Disk diffusion assay*

Two hundred microliter of the cultures of the test bacteria (1.5 $\times 10^8$ CFU/mL) was inoculated on to MHA medium. Presterilized 6 mm filter paper disks were soaked in each concentration of the extract and essential oil and carefully put on the agar media inoculated with the test bacteria. Tobramycin 10 µg/mL and DMSO 10% were used as positive and negative controls, respectively. The plates were subsequently incubated at 37 °C for 24h. After incubation, the growth inhibition zones were recorded using a transparent ruler (mm) and the readings were interpreted as mean value ± standard deviation.

2.6.2. *Minimum inhibitory concentration (MIC)*

Extract samples that showed significant antimicrobial activity with disk diffusion assay were selected to determine MIC using the broth dilution method. Four different concentrations were made from the extract and the oil using two-fold serial dilutions technique by transferring

appropriate concentrations from the stock solutions. The obtained concentrations were incubated with 0.02 mL bacterial cultures adjusted to 1.5 x 10^8 CFU/mL, and incubated at 37 ^{o}C for 24h. The growth of the bacteria was assessed by comparing the turbidity of the nutrient broth that contains plant extract and bacterial strains, with the controls. The smallest concentration where no turbidity was seen was considered as the extract's MIC value (Khalil *et al*., 2010; Radojevic *et al*., 2012).

2.6.3. *Minimum bactericidal concentrations (MBC)*

A loopful of the broth was taken from the test tubes in which growth was not visually detected and was streaked on nutrient agar (Muller Hinton Agar). The growth of bacterial cells was determined after 24 h incubation period at 37 °C. MBC was considered as the concentration of samples that didn't support the growth of cells on a fresh medium.

2.7. Antioxidant Assay

The antioxidant activity of the sample was evaluated by using DPPH assay. The crude extracts obtained using methanol, chloroform, and ethyl acetate were dissolved in four set of vials containing methanol to give 1000, 500, 250, and 125 µg/mL. While ascorbic acid was used as a positive control, a sample-free DPPH solution in methanol was utilised as a negative control. Four millilitres of DPPH solution (0.04 mg/mL) was mixed with 1 mL of extract preparations and then incubated for 30 min at room temperature. The absorbance of the samples was measured at 517 nm and the radical scavenging activitis were interpreted as present inhibition (IP) of free radicals using the formula:

$$IP\ (\%) = ([A_{control} - A_{test})/A_{control}]) \times 100\%.$$

Where $A_{control}$ is the absorbance of the control reaction, and A_{test} is the absorbance of the extracts (Suprava, 2012). The extracts' radical scavenging activity was also determined based on the percentage of the DPPH reduction by calculating the IC50 values.

2.8. Data Analysis

Microsoft excel and SPSS version 22 software were used to analyse the antibacterial activity of the crud extracts and essential oils against the tested bacterial species. The results were presented using mean value ± standard deviation

3. RESULTS and DISCUSSION

3.1. Phytochemical Screening

The phytochemical screening of methanol extract was positive for all tested phytochemicals and showed the existence of tannins, flavonoids, saponins, alkaloids, phenol, terpenoids, and steroids. However, the phenol and tannin constituents were absent in chloroform, and alkaloids were absent in ethyl acetate extracts (Table 1). Previous report (Mursyida *et al*., 2021) showed the presence of tannins, flavonoids, saponins, alkaloids, and essential oils, in *L. nobilis* leaf ethanol extract, which is consistent with the finding of this study. The result of this study was also compatible with the finding of Zuraida, 2018 and Algabri *et al*., 2018 who reported the availability of bioactive secondary metabolites in the leaf extracts of *L.nobilis*. Furthermore, the study of Onuminya *et al*. (2017) showed methanol extract of *L. nobilis* contains active compounds similar to those reported in the current study, except tannin. Variations in the composition of the secondary metabolites might be due to the difference in the genetics of the plant, climatic conditions of the environment of the plants, and the part of the plant examined (Dewijanti *et al*., 2019).

Table 1. Phytochemical compositions of *L. nobilis* leaf extracts.

Phytochemical Screening	Extracts		
	Methanol	Chloroform	Ethyl acetate
Alkaloids	+	+	_
Flavonoids	+	+	+
Saponins	+	+	+
Phenol	+	_	+
Tannin	+	_	+
Terpenoids	+	+	+
Steroids	+	+	+

Key: (+) presences (-) absences

3.2. *Laurus nobilis* EOs

The chemical content of the EOs from *L. nobilis* leaves are listed in Table 2. Investigation of the EOs by GC-MS (Figure 1) revealed 48 chemicals contributed for 91.4 % of the total compositions. The major constitutes were 2-Oxabicyclo[2.2.2]octane, 1,3,3-trimethyl-(2-Hydroxy-1,8-cineole) (26.3%), 3-Cyclohexene-1-methanol,.α.,.α.,4-trimethyl, acetate (α-terpinyl acetate) (17.1%), Benzene, 1,2-dimethoxy-4-(2-propenyl)-(Methylisoleugenol) (9.1%), α.-Terpineol [.α.,α.,4-trimethyl-3-cyclohexene-1-methanol] (5.2%), and 1,6-Octadien-3-ol, 3,7-dimethyl- -(Linalool) (4.6 %). The remaining constituents ranged from 0.3 to 3.5%. In general, the plant is composed of monoterpenes and sesquiterpenes or their derivatives.

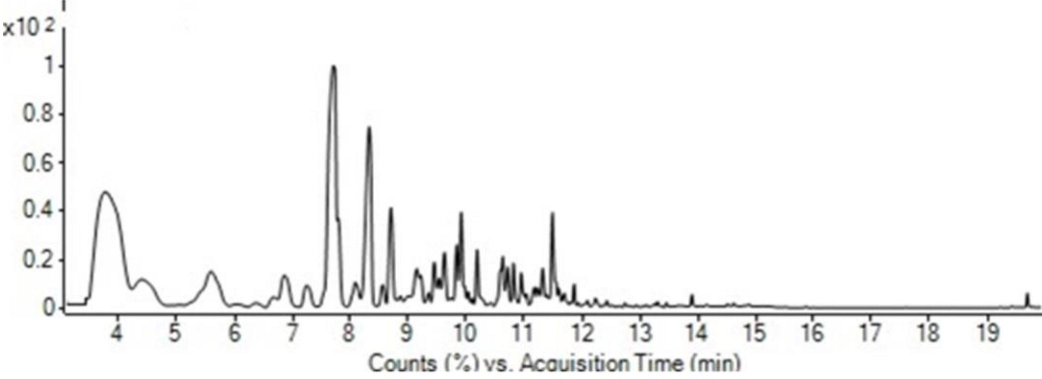

Figure 1. GC-MS chromatogram of essential oil of leaves of *L. nobilis.*

The comparison of chemical content of the oil of *L. nobilis* obtained in this study showed appreciable variation in 1,8-cineole percentage. The value of this finding (26.3%) is lower than the reported values of 44.97%, 56.0%, 58.59%, 52.43% and 34.62% from Türkiye, Tunisia, Cyprus, Morocco, and Algeria respectively (Sıdıka *et al*., 2013; Snuossi *et al*., 2016; Yalçın *et al*., 2007; Nabila *et al*., 2022; Mediouni Ben Jemâa *et al*., 2012). The amount of α-terpinyl acetate obtained in this study (17.1%) was greater than the value obtained by Fidan *et al*. (2019) (14.4%). However, it was comparable with the values reported previously by some investigators (Mediouni Ben Jemâa *et al*., 2012).

Essential oils of *L. nobilis* obtained from various locations were investigated by several evaluators to identify the chemical components of the plant. The results of their studies showed 1,8-cineole was the dominant component with a range of 26.70% to 68.48% (Özcan & Chalchat, 2005). Moreover, α-terpinyl acetate varies from 0.65-25.70% (Sangun *et al*., 2007; Sellami *et al*., 2011) and terpinen-4-ol from 1.50-4.56% (Di Leo Lira *et al*., 2009; Sellami *et al*., 2011) was found as major components. According to this aforementioned studies, the amount of 1, 8-cineole and α-terpinyl acetate recorded in this current study was compatible with previous reported amounts.

Table 2. GC-MS analysis result of EOs of *L.nobilis.*

Compound Name	RT	Formula	%
2(10)-Pinene	3.168	$C_{10}H_{16}$	1.3
2-Oxabicyclo[2.2.2]octane,1,3,3-trimethyl-(2-Hydroxy-1,8-cineole)	3.763	$C_{10}H_{18}O$	26.3
1,6-Octadien-3-ol, 3,7-dimethyl-(Linalool)	4.392	$C_{10}H_{18}O$	4.6
α-Terpineol[.α.,α.,4-trimethyl-3-cyclohexene-1-methanol]	5.605	$C_{10}H_{18}O$	5.2
3-isopropyl-6-methyl-7-oxabicyclo[4.1.0]hept-4-ene	6.685	$C_{10}H_{16}O$	0.5
1,7,7-Trimethylbicyclo[2.2.1]hept-2-yl acetate	6.87	$C_{12}H_{20}O_2$	2.3
3-Cyclohexene-1-methanol,.α.,.α.,4-trimethyl-, acetate (α-terpinyl acetate)	7.713	$C_{12}H_{20}O_2$	17.1
Eugenol	7.788	$C_{10}H_{12}O_2$	2.3
Copaene	8.089	$C_{15}H_{24}$	1.3
Benzene, 1,2-dimethoxy-4-(2-propenyl)-	8.325	$C_{11}H_{14}O_2$	9.1
1,1,7-Trimethyl-4-methylenedecahydro-1H-cyclopropa[e]azulene	8.702	$C_{15}H_{24}$	3.5
(1R,3aS,8aS)-7-Isopropyl-1,4-dimethyl-1,2,3,3a,6,8a-hexahydroazulene	9.007	$C_{15}H_{24}$	0.5
(-)-Germacrene D	9.452	$C_{15}H_{24}$	1.3
β-Selinene	9.532	$C_{15}H_{24}$	0.7
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.α.,4a.β.,8a.α.)-	9.839	$C_{15}H_{24}$	1.6
δ-Cadinene	9.919	$C_{15}H_{24}$	2.4
3a(1H)-Azulenol,2,3,4,5,8,8a-hexahydro-6,8a-dimethyl-3-(1-methylethyl)-,[3R-(3.α.,3a.α.,8a.α.)]-	9.971	$C_{15}H_{26}O$	0.3
(3S,3aR,3bR,4S,7R,7aR)-4-Isopropyl-3,7-dimethyloctahydro-1H-cyclopenta[1,3]cyclopropa[1,2]benzen-3-ol	10.041	$C_{15}H_{26}O$	0.3
Benzene,1,2,3-trimethoxy-5-(2-propenyl)-(Methylisoleugenol)	10.191	$C_{12}H_{16}O_3$	1.3
α.-Santalol	10.254	$C_{15}H_{24}O$	0.3
1H-Cycloprop[e]azulen-7-ol,decahydro-1,1,7-trimethyl-4-methylene-,[1ar-(1a.α.,4a.α.,7.α.,7a.β.,7b.α.)]-	10.636	$C_{15}H_{24}O$	1.8
1,1,7-trimethylspiro[2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa[e]azulene-4,2'-oxirane]	10.722	$C_{15}H_{24}O$	1.1
1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulen-4-ol	10.82	$C_{15}H_{26}O$	1
γ.-HIMACHALENE	11.178	$C_{15}H_{24}$	0.6
Guaiol	11.236	$C_{15}H_{26}O$	0.5
.tau.-Cadinol	11.323	$C_{15}H_{26}O$	0.9
2-Naphthalenemethanol,decahydro-.α.,.α.,4a,8-tetramethyl-,didehydroderiv.,[2R-(2.α.,4a.α.,8a.β.)]-	11.49	$C_{15}H_{26}O$	2.5
2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	11.698	$C_{15}H_{24}O$	0.4
(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	11.866	$C_{15}H_{24}O$	0.4
Total			91.4

3.4 Biological Activity of Crude Extract and Essential Oil

3.4.1 Antibacterial activity of L. nobilis

The extract and the EO of the *L. nobilis* were evaluated at four concentration levels against two Gram-positive and four Gram-negative bacteria using tobramycin and DMSO as a positive and negative controls respectively. Overall antibacterial activities of *L. nobilis* extract inhibition zone ranged from 7.00± 0.00mm to 16.0±1.00mm (Table 3). The maxim inhibition zone was recorded by the ethyl acetate extract against *S. typhimurim* (16.0±1.00 mm). While the chloroform extract showed highest inhibition against *E. coli* (14.33±1.53mm), and methanol showed high activity on *S. aureus* (13.67±0.58mm) at 200 mg/mL compared to tobramycin (16.90±0.80mm and 19.16±0.58mm respectively) at 10 µg/mL. However, the crude extract of methanol showed no inhibiton against *K. pneumonia, S. typhimurium and E. faecalis,* and the chloroform extract showed no inhibit against *E. faecalis* at a concentration of 25-200 mg/mL, respectively (Table 3).

In another study, the extracts of *L. nobilis* were found to be prominently active against *B. subtilis, S. aureus, S. epidermidis*, *E. coli* and *P. aeruginosa* at concentrations less than 5 mg/mL

(Algabri *et al*., 2018), and the methanol extract of bay leaves showed inhibition against *S. aureus* (18 ± 0.8 mm). In contrast, there was no antibacterial activity against bacteria like *P. aeruginosa and E. coli* (Algabri *et al*., 2018). The difference may be due to multipolar factors such as the type and origin of the *L. nobilis*, drying and extraction methods, and differences in the crude extract concentration.

Table 3. Antibacterial activities of extracts from *Laurus nobilis*.

Extract type	Conc. (mg/mL)	Inhibition Diameter (mm)±*SD*					
		E. coli	*P. aeruginosia*	*K. pneumonia*	*S. typhimurium*	*E. faecalis*	*S. aureus*
Methanol	200	NI	9.00±0.00	NI	NI	NI	13.67±0.58
	100	NI	8.67±0.58	NI	NI	NI	13.33±1.52
	50	NI	7.00±0.00	NI	NI	NI	12.33±0.58
	25	NI	7.00±0.00	NI	NI	NI	12.67±0.58
Chloroform	200	14.33±1.53	13.0±0.00	14.00±0.00	11.67±1.15	NI	12.33±1.53
	100	14.00±1.00	12.67±0.58	12.67±0.57	11.33±0.58	NI	11.0±1.73
	50	12.67±0.58	12.0±1.0	11.67±1.15	10.33±0.58	NI	10.0±1.73
	25	11.33±0.58	8.0±1.0	6.33±0.58	8.67±1.15	NI	9.0±1.73
Ethyl acetate	200	14.33±0.58	14.0±0.00	13.0±0.00	16.0±1.00	14.67±0.58	13.33±1.53
	100	14.33±0.58	13.0±1.00	13.0±0.00	15.33±1.53	13.67±0.58	13.33±1.53
	50	14.0±1.00	12.33±0.58	11.67±0.58	14.0±1.00	13.0±0.00	8.33±0.58
	25	14.0±1.00	11.67±0.58	10.67±1.15	13.0±1.00	12.33±2.08	7.33±0.58
Tobramycin	10µg/mL	16.90±0.80	20.84±0.80	11.33±0.80	17.30±0.50	13.83±1.15	19.16±0.58
Negative control		6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00

Key: NI= No Inhibition

The essential oil of *L. nobilis* demonstrated antibacterial activities against both types of bacteria at 0.045 mg/mL. Among this, maximum zone of inhibition (12.33±0.58 mm) was measured in *P. aeruginosa* and *K. pneumonia.* The positive control tobramycin showed an inhibitory zone of 20.00 ± 1.73 mm and 12.33 ± 0.58 mm against the tested bacteria pathogens, respectively. The antibacterial investigation of this work revealed the activities of the extracts and the oil of *L. nobilis* (Tables 3 and 4) increase with increased concentration. This observation agrees with previously reported findings, and antibacterial activities were increased with increasing the concentration of extract (Jahangirian *et al*., 2013).

Different types of antimicrobial testing method can be used to evaluate the biological activities of plants and their constituents. The variation of the method may influence the inhibition levels. Additionally, other factors like season of sample collection, plant part analysed, and the composition within the plant material may result in the differences of antimicrobial activity of plant material (Fidan *et al*., 2019). The three extracts of *L.nobilis* obtained in this study exhibited different zone of inhibition towards the tested bacteria (Table 3). This difference may be due to the variation of the solvents used to extract the plant material. The extract obtained by using ethyl acetate as an extraction solvent showed the best activity compared to others. The antibacterial activity of *L. nobilis* EO may be attributed to its ability to disrupt cell membrane and affect the semipermeable property of bacterial cells. The oil may also alter the proteins attached to the cell membrane and disturb the transport of nutrients in and out of the cell. Previous reports showed that terpenes are the components accounted for the antibacterial activity of *L. nobilis* essential oil (Siriken *et al*., 2018). Similar to this study finding, the main component of *L. nobilis* oil (1, 8-cineole) has been also implicated in previous studies, inhibited the growth of several microorganisms (Caputo *et al*., 2017).

The variation of inhibition zones displayed by different group of bacteria attributed primarily to the difference chemical composition of the sample and morphology of the bacterial cell membrane. The pronounced antibacterial activity seen in Gram-positive bacteria might be because of the peptidoglycan layer permeating the hydrophobic components in the extract (Rameshkumar *et al*., 2007). The resistance in Gram-negative bacteria could be due to the lipopolysaccharide layer in the cell membrane. The outer membrane of Gram-negative bacteria is made up of hydrophobic lipopolysaccharides, efficient in resisting the entry of hydrophilic compounds (Zgurskaya *et al*., 2015).

In this study, the EOs of *L. nobilis* exhibits greater inhibition effect towards Gram-negative bacteria (Table 4). This finding did not agree with the previous report (Fidan *et al*., 2019) that claimed that Gram-positive bacteria are generally more susceptible to the oils' action than Gram-negative ones. Contrary to our current finding, the EO of *L. nobilis* didn't show inhibitory activity against *P. aurognosa* and *E. coli*. The main EOs constituent of this *L. nobilis* was 1,8-cineole, which has shown good inhibition against many microorganisms, and this constituent has also been reported by previous studies (Caputo *et al*., 2017). Each EO contains a varied mixture of constituents that may play a role in the extended spectrum of antimicrobial activity.

Table 4. Antibacterial activities of essential oil from *L. nobilis*.

Compound	Conc. (mg/mL)	Inhibition Diameter (mm)±*SD*					
		E. coli	*P. aeruginosia*	*K. pneumonia*	*S.typhimurium*	*E. faecalis*	*S.aureus*
Essential oil	0.045	12.33±1.15	12.33±0.58	12.33±0.58	NI	11.33±1.15	11.67±1.15
	0.022	10.60±0.58	11.67±0.58	10.33±0.58	NI	9.33±1.53	10.67±1.15
	0.011	10.33±0.58	10.00±0.00	10.33±1.15	NI	7.00±0.00	7.00±0.00
	0.005	10.00±0.00	9.00±0.00	9.67±1.52	NI	7.0±0.00	7.00±0.00
Tobramycin	10µg/mL	16.67±1.15	20.00±1.73	12.33±0.58	14.33±1.53	10.67±0.58	20.00±1.00
Negative control	10% DMSO	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00

Key: NI=No Inhibition

3.4.2 The MIC and MBC

The MIC value for the EtOAc and chloroform extracts was 2.5 mg/mL (except *P. arognosa* and *S. aureus*, 5 mg/mL) (Table 5). The MIC was 1.25 mg/mL for the Methanol extract and 0.11 mg/mL for the essential oil against all the tested bacteria except for *S. aureus* (0.22 mg/mL). *Ethyl acetate and chloroform* extract revealed bactericidal activity against the tested organisms with MBC of 5 mg/mL. However, chloroform showed MBC against *P. arognosa* and *S. aureus* at 10 mg/mL. In contrast, the MBC of methanol extract was 2.5 mg/mL against all of the tested bacteria. The MBC results for the EO was 0.22 mg/mL except for *S. aureus*, which was less sensitive, and its minimal bactericidal concentration was 0.44 mg/mL (Table 6).

Table 5. The MIC and MBC values for the extracts in mg/mL.

Bacteria	Methanol		Chloroform		Ethyl acetate	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	_	_	2.5	5	2.5	5
P. aeruginosia	1.25	2.5	5	10	2.5	5
K. pneumonia	_	_	2.5	5	2.5	5
S.typhimurium	_	_	2.5	5	2.5	5
E. faecalis	_	_	_	_	2.5	5
S. aureus	1.25	2.5	5	10	2.5	5

Table 6. The MIC and MBC values of the EO in mg/mL.

Bacteria	Essential oil	
	MIC	MBC
E. coli	0.11	0.22
P. aeruginosia	0.11	0.22
K. pneumonia	0.11	0.22
S.typhimurium	NA	NA
E. faecalis	0.11	0.22
S. aureus	0.22	0.44

3.4.3 Antioxidant activity of L. nobilis

The DPPH radical scavenging activities of the crude extracts (%) were 94.75, 89.12, and 96.11 for the ethyl acetate, chloroform and methanol extracts, respectively (Table 7). Among the studied crude samples MeOH extract of 1000 µg/mL had the same radical scavenging activity with the standard ascorbic acid (96.11). For the EO the free radical scavenging activity was 24.40 at 100 µg/ml (Table 8). Our finding implied the activity of the extracts against DPPH radical increase with increased dosage or concentration of the extract (Elmastaş *et al*., 2006).

IC_{50} values for the ethyl acetate, chloroform, and methanol extracts obtained in this study were 45.5, 153.2, and 0.02, respectively. Since low IC_{50} values correspond to a higher antioxidant capacity, compared to previous studies, the value obtained in this study (45.5) is better than the earlier values (75.65 µg/mL and 83.24 µg/mL) reported by Conforti *et al*., 2016. The effective antioxidant activities of *L. nobilis* seen in this study ars related to the phenolic compounds present in the plant (Renuka *et al*., 2018) which have a high degree of hydroxylation, manifested in the increased ability to donate protons and hence, stabilize the DPPH radical. A specific correlation of the antioxidant properties and the amount of phenols and flavonoids existed in the plant material was reported by some investigators (Larissa da Silva *et al*., 2017). *L. nobilis* leaves are good source of natural phenols and flavonoids which have antioxidant activists that benefit public health. Indeed, using these substances helps to minimize chemical products which usually initiate some secondary undesirable impacts (Taroq *et al*., 2018).

Table 7. Antioxidant activities of crude extract from *L. nobilis.*

Conc. (µg/mL)	Control	Crude Extracts						Positive control	
		Ethyl acetate		Chloroform		Methanol		Ascorbic Acid	
		A	%RSA	A	%RSA	A	%RSA	A	%RSA
1000	1.029	0.054	94.75	0.112	89.12	0.04	96.11	0.04	96.11
500	1.029	0.055	94.66	0.279	72.89	0.049	95.24	0.045	95.63
250	1.029	0.181	82.41	0.428	58.41	0.056	94.56	0.047	95.43
125	1.029	0.358	65.21	0.543	47.23	0.142	86.2	0.05	95.14
IC_{50}		45.5		153.2		0.02			

According to Ibrahim *et al*., 2020, the potency of *L. nobilis* EOs to scavenge free radicals is attributed to the components present in the oil (Cherrat *et al*., 2014), particularly to the high proportion of 1.8-cineole (26.3%) and α-terpinyl acetate (17.1%). Furthermore, other compounds such as methylisoleugenol (9.1%), α.-terpineol (5.2%) and linalool (4.6 %) may be involved. Our results are aligned with previous findings (Celikel & Kavas, 2008; Cherrat *et al*., 2014), and further confirm the antioxidant capacity of EOs from *L. nobilis* and its potential as a natural preservative in food and pharmaceutical industries.

Reports showed that oxidative stress, which occurs when free radical generation in the body exceeds the the defines system, the biological basis of chronic conditions such as arteriosclerosis can be formed (Elmastaş *et al*., 2006). Oxidative stress is recognized as a disproportionate production of reactive species compared to the antioxidant defines system and

according to some studies (Carocho & Ferreira, 2013) an enhanced production of the antioxidants has direct association with decreased degenerative diseases including cancer, diabetes, neurodegenerative and cardiovascular diseases. This study revealed the crude extracts and essential oils showed free radical inhibition activity and may limit free radical damage occurring in the human body.

Table 8. Antioxidant activities of the EO from *L. nobilis,*

Concentration (µg/mL)	Control	Essential oil		Positive control Ascorbic acid	
		A	%RSA	A	%RSA
100	1.275	0.964	24.4	0.028	97.8
50	1.275	1.027	19.45	0.028	97.8
25	1.275	1.059	16.94	0.032	97.49
12.5	1.275	1.086	14.82	0.035	97.25
IC_{50}		339.96			

4. CONCLUSION

This study showed the antibacterial and antioxidant activities of crude extracts and essential oil of *L.nobilis* on different bacterial strains through an in vitro experiment appeared exciting and promising. The crude extract of ethyl acetate showed the highest zone of inhibition against *S. thyphimurium* (16.00± 1.00mm) at 200 mg/mL compared to the positive control tobramycin (17.30±0.50mm) at 10 µg/mL. GC-MS analysis of essential oils obtained from *L.nobilis* leaves revealed a total of 48 chemicals accounted for 91.4 % of the whole. The major constitutes were 2-oxabicyclo[2.2.2]octane, 1,3,3-trimethyl- (2-hydroxy-1,8-cineole) (26.3%), 3-cyclohexene-1-methanol, .α.,.α.,4-trimethyl-, acetate (α-terpinyl acetate) (17.1%), benzene, 1,2-dimethoxy-4-(2-propenyl)- (methylisoleugenol) (9.1%), α.-terpineol [.α.,α.,4-trimethyl-3-cyclohexene-1-methanol](5.2%), and 1,6-octadien-3-ol, 3,7-dimethyl- -(linalool) (4.6 %). *L.nobilis* EOs showed maximum inhibition against *P. aeruginosa and K. pnumonia* (12.33±0.58mm) at 0.045 mg/mL compared to tobramycin (24.0±1.73 and 12.33±0.58mm) respectively at 10 µg/mL. The finding of this study showed that bay leaf has considerable antimicrobial and antioxidant properties.

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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

Rebecca Beyene: accomplihsed the laboratory experimental work and prepared the original draft manuscript. **Teshome Geremew** and **Aman Dekebo** supervised the experimental work and edited the manuscript.

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REFERENCES

Algabri, S.O., Doro, B.M., Abadi, A.M., Shiba, M.A., & Salem, A.H. (2018). Bay Leaves have Antimicrobial and Antioxidant Activities. *International Journal of Pathogen Research, 1*(1), 3.

Andrews, J.M. (2002). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *49*(6), 1049–1049. https://doi.org/10.1093/jac/dkf083

Archana, D., Dixitha, M., & Santhy, K.S. (2015). Antioxidant and anti clastogenic potential of Piper longum L. *International Journal of Applied Pharmaceutics*, *7*(2), 11–14.

Baser, K.H.C., & Buchbauer, G. (Eds.). (2015). *Handbook of Essential Oils*. CRC Press. https://doi.org/10.1201/b19393

Batool, S., Khera, R.A., Hanif, M.A., & Ayub, M.A. (2019). Bay Leaf. *Medicinal Plants of South Asia: Novel Sources for Drug Discovery*, *January*, 63-74. https://doi.org/10.1016/B978-0-08-102659-5.00005-7

Bounatirou, S., Smiti, S., Miguel, M.G., Faleiro, L., Rejeb, M.N., Neffati, M., Costa, M.M., Figueiredo, A.C., Barroso, J.G., & Pedro, L.G. (2007). Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. *Food Chemistry*, *105*(1), 146-155. https://doi.org/10.1016/j.foodchem.2007.03.059

Caputo, L., Nazzaro, F., Souza, L.F., Aliberti, L., De Martino, L., Fratianni, F., Coppola, R., & De Feo, V. (2017). *Laurus nobilis:* Composition of essential oil and its biological activities. In *Molecules* (Vol. 22, Issue 6). https://doi.org/10.3390/molecules22060930

Carocho, M., & Ferreira, I. (2013). The Role of Phenolic Compounds in the Fight against Cancer – A Review. *Anti-Cancer Agents in Medicinal Chemistry*, *13*(8), 1236–1258. https://doi.org/10.2174/18715206113139990301

Celikel, N., & Kavas, G. (2008). Antimicrobial properties of some essential oils against some pathogenic microorganisms. *Czech Journal of Food Sciences*, *26*(3), 174–181. https://doi.org/10.17221/1603-cjfs

Cherrat, L., Espina, L., Bakkali, M., García-Gonzalo, D., Pagán, R., & Laglaoui, A. (2014). Chemical composition and antioxidant properties of *Laurus nobilis* L. and *Myrtus communis* L. essential oils from Morocco and evaluation of their antimicrobial activity acting alone or in combined processes for food preservation. *Journal of the Science of Food and Agriculture*, *94*(6), 1197–1204. https://doi.org/10.1002/jsfa.6397

Conforti, F., Statti, G., Uzunov, D., & Menichini, F. (2016). Comparative Chemical Composition and Antioxidant Activities of Wild and Cultivated *Laurus nobilis* L. Leaves and *Foeniculum vulgare* subsp. *piperitum* (Ucria) Coutinho Seed*s* (pp. 2056–2064). Leaves and *Foeniculum vulgare* Biological and Pharmaceutical Bulletin.

Delcour, A.H. (2009). Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta*, *1794*(5), 808–816. https://doi.org/10.1016/j.bbapap.2008.11.005

Dewijanti, I.D., Mangunwardoyo, W., Artanti, N., & Hanafi, M. (2019). Bioactivities of Salam leaf (*Syzygium polyanthum* (Wight) Walp). *AIP Conference Proceedings*, *2168*(November). https://doi.org/10.1063/1.5132499

Di Leo Lira, P., Retta, D., Tkacik, E., Ringuelet, J., Coussio, J.D., van Baren, C., & Bandoni, A.L. (2009). Essential oil and by-products of distillation of bay leaves (*Laurus nobilis* L.) from Argentina. *Industrial Crops and Products*, *30*(2), 259-264. https://doi.org/10.1016/j.indcrop.2009.04.005

Elmastaş, M., Gülçin, I., Işildak, Ö., Küfrevioğlu, Ö.I., Ibaoğlu, K., & Aboul-Enein, H.Y. (2006). Radical scavenging activity and antioxidant capacity of bay leaf extracts. *Journal of the Iranian Chemical Society*, *3*(3), 258–266. https://doi.org/10.1007/BF03247217

Fang, F., Sang, S., Chen, K.Y., Gosslau, A., Ho, C.T., & Rosen, R.T. (2005). Isolation and identification of cytotoxic compounds from Bay leaf (*Laurus nobilis*). *Food Chemistry*, *93*(3), 497–501. https://doi.org/10.1016/j.foodchem.2004.10.029

Fidan, H., Stefanova, G., Kostova, I., Stankov, S., Damyanova, S., Stoyanova, A., & Zheljazkov, V. D. (2019). Chemical Composition and Antimicrobial Activity of *Laurus nobilis* L. Essential oils from Bulgaria. *Molecules*, *24*(4), 1-10. https://doi.org/10.3390/molecules24040804

Fukuyama, N., Ino, C., Suzuki, Y., Kobayashi, N., Hamamoto, H., Sekimizu, K., & Orihara, Y.

(2011). Antimicrobial sesquiterpenoids from *Laurus nobilis* L. *Natural Product Research*, *25*(14), 1295–1303. https://doi.org/10.1080/14786419.2010.502532

Hanuš, L.O., Rosenthal, D., Řezanka, T., Dembitsky, V.M., & Moussaief, A. (2008). Fast and easy GC/MS identification of myrrh resins. *Pharmaceutical Chemistry Journal*, *42*(12), 719–720. https://doi.org/10.1007/s11094-009-0209-z

Ibrahim, M., Abdelkrim, A., Asmae, E.G., Noureddine, H., Badiaa, L., & Elhoussine, D. (2020). Chemical Composition, Antioxidant Activity, and Antifungal Effects of Essential Oil from *Laurus nobilis* L. *Flowers Growing in Morocco*, *20*.

Jahangirian, H., Haron, J., Shah, M., & Al, E. (2013). Well Diffusion Method for Evaluation of Antibacterial Activity. *Digest Journal of Nanomaterials and Biostructures*, *8*(3), 1263–1270.

Kaurinovic, B., Popovic, M., & Vlaisavljevic, S. (2010). In vitro and in vivo effects of *Laurus nobilis* L. leaf extracts. *Molecules*, *15*(5), 3378-3390. https://doi.org/10.3390/molecules15053378

Khalil, E., Elkhair, A., & Mohsen, U.A. (2010). *Antibacterial Activity and Phytochemical Analysis of Some Medicinal Plants from Gaza Strip-Palestine Post treatment of secondary wastewater effluent using algae View project Waste treatment View project*. *January*.

Larissa da Silva, I., Karuppusamy, A., Fabio, M., Ivana Maria Povoa, V., Isanete Geraldini Costa, B., Sikiru Olaitan, B., & Domingos Tabajara de Oliveira, M. (2017). Antimicrobial and antioxidant activities of selected plants used by populations from Juruena Valley , Legal Amazon , Brazil. *International Journal of Pharmacy and Pharmaceutical Sciences*, *9*(5), 179–191.

Mediouni Ben Jemâa, J., Tersim, N., Toudert, K.T., & Khouja, M.L. (2012). Insecticidal activities of essential oils from leaves of *Laurus nobilis* L. from Tunisia, Algeria and Morocco, and comparative chemical composition. *Journal of Stored Products Research*, *48*, 97–104. https://doi.org/10.1016/j.jspr.2011.10.003

Mursyida, E., Almira, R., Widiasari, S., & Misfa, O. (2021). Antibacterial Activity of Bay Leaf (*Syzygium polyanthum*) Ethanol Extract on *Escherichia coli* Growth. *Photon: Jurnal Sain Dan Kesehatan*, *12*(1), 12–18. https://doi.org/10.37859/jp.v12i1.3142

Nabila, B., Piras, A., Fouzia, B., Falconieri, D., Kheira, G., Fedoul, F.F., & Majda, S.R. (2022). Chemical composition and antibacterial activity of the essential oil of *Laurus nobilis* leaves. *Natural Product Research*, *36*(4), 989-993. https://doi.org/10.1080/14786419.2020.1839450

Onuminya, T., Shodiya, O., & Olubiy, O. (2017). Comparative proximate and phytochemical analyses of leafy vegetables in lagos state. *Nigerian Journal of Pure and Applied Sciences*, *30*(3), 3097–3103.

Özcan, M., & Chalchat, J.-C. (2005). Effect of Different Locations on the Chemical Composition of Essential Oils of Laurel (*Laurus nobilis* L.) Leaves Growing Wild in Turkey. *Journal of Medicinal Food*, *8*(3), 408–411. https://doi.org/10.1089/jmf.2005.8.408

Parthasarathy, V.A., Chempakam, B., & Zachariah, T.J. (2008). Chemistry of spices. In *Chemistry of Spices*. https://doi.org/10.4327/jsnfs1949.32.267

Radojevic, I.D., Stankovic, M.S., Stefanovic, O.D., Topuzovic, M.D., Comic, L.R., & Ostojic, A.M. (2012). Great horsetail (*Equisetum telmateia* Ehrh.): Active substances content and biological effects. *EXCLI Journal*, *11*, 59–67.

Rameshkumar, K.B., George, V., & Shiburaj, S. (2007). Chemical constituents and antibacterial activity of the leaf oil of *Cinnamomum chemungianum* Mohan et Henry. *Journal of Essential Oil Research*, *19*(1), 98-100. https://doi.org/10.1080/10412905.2007.9699238

Renuka, K., Devi, V.R., & Subramanian, S.P. (2018). Phytochemical screening and evaluation of in vitro antioxidant potential of immature Palmyra Palm (*Borassus flabellifer* Linn.) Fruits. *International Journal of Pharmacy and Pharmaceutical Sciences*, *10*(8), 77. https://doi.org/10.22159/ijpps.2018v10i8.27162

Saeed, S., & Tariq, P. (2007). Antimicrobial activities of *Emblica officinalis* and *Coriandrum sativum* against gram positive bacteria and *Candida albicans. Pakistan Journal of Botany*, *39*(3), 913–917.

Sahoo, S. (2012). Evaluation of in vitro antioxidant activity of leaf extract of *Alpinia malaccensis. Journal of Medicinal Plants Research*, *6*(23), 4032-4038. https://doi.org/10.5897/jmpr12.374

Sangun, M.K., Aydin, E., Timur, M., Karadeniz, H., Caliskan, M., & Ozkan, A. (2007). Comparison of chemical composition of the essential oil of *Laurus nobilis* L. leaves and fruits from different regions of Hatay, Turkey. *Journal of Environmental Biology*, *28*(4), 731–733.

Santoyo, S., Lloría, R., Jaime, L., Ibañez, E., Señoráns, F.J., & Reglero, G. (2006). Supercritical fluid extraction of antioxidant and antimicrobial compounds from *Laurus nobilis* L. Chemical and functional characterization. *European Food Research and Technology*, *222*(5–6), 565–571. https://doi.org/10.1007/s00217-005-0027-9

Sellami, I.H., Wannes, W.A., Bettaieb, I., Berrima, S., Chahed, T., Marzouk, B., & Limam, F. (2011). Qualitative and quantitative changes in the essential oil of *Laurus nobilis* L. leaves as affected by different drying methods. *Food Chemistry*, *126*(2), 691–697. https://doi.org/10.1016/j.foodchem.2010.11.022

Sharmeen, J.B., Mahomoodally, F.M., Zengin, G., & Maggi, F. (2021). Essential oils as natural sources of fragrance compounds for cosmetics and cosmeceuticals. *Molecules*, *26*(3). https://doi.org/10.3390/molecules26030666

Siriken, B., Yavuz, C., & Guler, A. (2018). Antibacterial Activity of *Laurus nobilis*: A review of literature. *Medical Science and Discovery*, *90*(438), 374-379. https://doi.org/10.17546/msd.482929

Sıdıka, E., Oktay, Y., Hatice, E.T., Aslı, A., & Merve, A.U. (2013). Chemical composition, antimicrobial activity and antioxidant capacity of some medicinal and aromatic plant extracts. *African Journal of Microbiology Research*, *7*(5), 383-388. https://doi.org/10.5897/AJMR12.1765

Snuossi, M., Trabelsi, N., Taleb, S. Ben, Dehmeni, A., Flamini, G., & De Feo, V. (2016). *Laurus nobilis, Zingiber officinale* and *Anethum graveolens* essential oils: Composition, antioxidant and antibacterial activities against bacteria isolated from fish and shellfish. *Molecules*, *21*(10). https://doi.org/10.3390/molecules21101414

Subba, B., Seling, T.R., Kandel, R.C., & Phuyal, G.P. (2017). Assessment of antimicrobial and antioxidant activities of *Amomum subulatum* Roxb. of Nepal. *Asian Journal of Pharmaceutical and Clinical Research*, *10*(4), 95-97. https://doi.org/10.22159/ajpcr.2017.v10i4.16018

Taroq, A., El Kamari, F., Aouam, I., El Atki, Y., Lyoussi, B., & Abdellaoui, A. (2018). Antioxidant activities and total phenolic and flavonoid content variations of leaf extracts of *Laurus nobilis* l. from Morocco. *Asian Journal of Pharmaceutical and Clinical Research*, *11*(12), 540–543. https://doi.org/10.22159/ajpcr.2018.v11i12.29747

Yalçın, H., Anık, M., Şanda, M.A., & Çakır, A. (2007). Gas Chromatography/Mass Spectrometry Analysis of *Laurus nobilis* Essential Oil Composition of Northern Cyprus. *Journal of Medicinal Food*, *10*(4), 715–719. https://doi.org/10.1089/jmf.2007.404

Zuraida, Z. (2018). Analisis Toksisitas Beberapa Tumbuhan Hutan Dengan Metode Brine Shrimp Lethality Test (Bslt). *Jurnal Penelitian Hasil Hutan*, *36*(3), 239–246. https://doi.org/10.20886/jphh.2018.36.3.239-246

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Research Article

Chemical content profile and antioxidant activity of *Rhododendron ponticum* L. (Ericaceae) extracts

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Abstract: *Rhododendron* species (Ericaceae) is considered the most diverse group, with more than 1200 species famous for their colorful flowers. *Rhododendron*, also known as azalea, is a flowering tree in the Ericaceae family. Herein, *in vitro* antioxidant activities of acetone and methanol extracts of *Rhododendron ponticum* leaves were studied along with their phenolic contents using High-Performance Liquid Chromatography with a Diode-Array Detector (HPLC-DAD). Antioxidant activity was performed spectrophotometrically using $ABTS^{\cdot+}$, DPPH·, CUPRAC, and β-carotene/linoleic acid assays. Acetone extract showed better antioxidant activity than methanol extract in all tests. The HPLC-DAD analysis revealed fifteen phenolic compounds, of which seven were common for both extracts. Catechin (25.80 and 33.08 mg/g extract, respectively) and epicatechin (31.15 and 26.54 mg/g extract, respectively) were calculated as major phenolic components in acetone and methanol extracts.

1. INTRODUCTION

Medicinal plants are the richest biological source of drugs for traditional medicine systems, modern drugs applied in clinical therapy, nutraceuticals, food supplements, folk remedies, cosmetics, nutricosmetics, pharmaceutical intermediates, and chemical assets for synthetic drugs. One of the essential areas in which developed countries contribute to the economy is the bulk trade of medicinal and aromatic plants. The first and most crucial step in adding value to natural products is the production of herbal medicine extracts using various methods, from traditional approaches to advanced technological extraction techniques. Together with the increasing demand for natural products that have medicinal properties for health all over the world, manufacturers of medicinal plant extracts have started to use the most appropriate extraction technologies to produce extracts of defined quality.

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In traditional medicines used around the world, different *Rhododendron* species are also known to have applications against various inflammatory conditions and pain (Chosson *et al*., 1998; Baytop, 1999; Li *et al*., 2000; Erdemoğlu *et al*., 2008). *Rhododendron ponticum* L., which is distributed in the northern parts of Türkiye, is a large evergreen deciduous shrub. It is a member of the Ericaceae family. There are five Rhododendron species and four hybrids in the Flora of Türkiye (Stevens, 1978; Sales and Milne, 2000). The sap obtained from the freshly cut branch of *R. ponticum* is used in Turkish folk medicine by dripping into the tooth cavity against toothache. In addition, after the plant's fresh leaves are dried in the fire, it is applied externally to the affected area for the treatment of back pain or joint edema. (Yeşilada *et al*., 1999). Oral treatment of 2% leaf infusion of *R. ponticum* alleviates rheumatic pains (Baytop, 1999). It has been reported that *Rhododendron* species exhibit many biological activities such as anti-inflammatory, antidiabetic, analgesic, antimicrobial, insecticidal and antioxidant upon its plant extracts and pure components (Oztasan *et al*., 2005; Erdemoğlu *et al*., 2008; Prakash *et al*., 2008; Jing *et al*., 2009; Silici *et al*., 2010; Yarlıoğlues *et al*., 2011).

It will direct our country, which is rich in medicinal and aromatic plants, to domestic production in the world health sector by contributing to the country's economy by encouraging the local people to grow medicinal and aromatic plants by bringing the plants whose extracts have been identified to be usable in the health sector to a better position in the country. In many studies, antioxidants can prevent or delay the oxidation of an oxidizable substrate in a chain reaction. Hence, they have a significant place in preventing many diseases. Antioxidant supplements, which have the possible potential to fight conditions linked to oxygen species by scavenging free radicals, are such as nutraceuticals, medicinal drinks, and syrup.

The antioxidant activities of phenolic compounds are associated with several different mechanisms, including free radical inhibition, hydrogen transfer, singlet oxygen removal, and metal ion chelation, and they act as a substrate for radicals such as superoxide and hydroxyl. A direct relationship was found between the phenolic content of plants and their antioxidant capacity (Robards *et al*., 1999; Al-Mamary *et al*., 2002; Caliskan *et al.,* 2022).

In this study, phenolic component analyses of the acetone and methanol extracts of *R. ponticum* leaves were screened against 42 reference materials using the HPLC-DAD instrument. Acetone and methanol extracts of *R*. *ponticum* leaves were also tested for antioxidants using four complementary methods (*β*-carotene/linoleic acid, DPPH$^{\bullet}$ scavenging, ABTS$^{\bullet+}$ scavenging, CUPRAC reducing assays). The relationship between phenolic contents and antioxidant activity between *R. ponticum* methanol and acetone extracts was investigated.

2. MATERIAL and METHODS

2.1. Plant Material Collection and Extraction

R. ponticum was obtained from Trabzon Akçaabat in September 2020 and compared with herbarium samples. *R. ponticum* leaves were dried in the laboratory under shade. Acetone and methanol extracts of *R. ponticum* were obtained using the maceration technique. Then, the solvents were removed using a rotary evaporator under vacuum to obtain crude extracts. The visual of *R. ponticum* is given in Figure 1.

Figure 1. The visual of *R. ponticum*

2.2. Chemical Content

2.2.1. *Determination of phenolic profiles by HPLC-DAD*

2.2.1.1. Preparation samples for HPLC-DAD analysis: 8 mg extracts dissolved in 1 mL methanol and was homogenized in an ultrasonic bath at 20°C for 5 min and filtered through 0.45 µm PTFE filters.

2.2.1.2. HPLC-DAD analysis: The phenolic profile of *R. ponticum* leaf extracts was screened with a modification of the method described by Tokul-Ölmez et al. 2020. In this study, methanol and acetone extracts were investigated against 42 standard compounds (fumaric acid, gallic acid, protocatechuic acid, theobromine, theophylline, catechin, 4-hydroxy benzoic acid, 6,7-dihydroxycoumarine, methyl-1,4-benzoquinone, vanillic acid, caffeic acid, vanillin, chlorogenic acid, *p*-coumaric acid, ferrulic acid, cynarin, coumarine, prophylgallate, rutin, *trans*-2-hydroxycinnamic acid, ellagic acid, myricetin, fisetin, quercetin, *trans*-cinnamic acid, luteoline, rosmarinic acid, kaempherol, apigenin, chrysin, 4-hydroxy resorcinol, 1,4-dichlorobenzene, pyrocatechol, 4-hydroxybenzaldehyde, epicatechin, 2,4-dihydroxybenzaldehyde, hesperedin, oleuropein, naringenin, hesperetin, genistein, curcumin) using a Shimadzu high-performance liquid chromatography (Shimadzu Cooperation, Japan) system that consists of a Shimadzu model LC-20AT. The column temperature was set at 35 ℃. The chromatographic separation was performed on a C_{18} (5 µm, 4.6 mm x 250 mm) reverse phase column and an Inertsil C_{18} guard column (Tokul-Ölmez *et al*., 2020).

2.3. *Antioxidant activity*

2.3.1. *Determination of the antioxidant activity with the β-carotene bleaching method*

The antioxidant activity of *R. ponticum* leaf extracts was evaluated using a *β*-carotene-linoleic acid assay (Miller, 1971). *β*-carotene (0.5 mg) in 1 mL of $CHCl_3$ was added to 25 µL of linoleic acid and 200 mg of Tween 40. As soon as $CHCl_3$ was evaporated under vacuum, distilled H_2O saturated with O_2 was added and shaken vigorously. 160 µL of this prepared mixture was added to 40 µL of extracts at different concentrations in a 96-well plate. After adding the emulsion to each, the zero-time absorbance was measured at 470 nm. The mixture was incubated for two hours at 37 °C. A blank, devoid of *β*-carotene, was prepared for background subtraction. BHT and α-TOC were used as standards.

2.3.2. *DPPH• scavenging activity assay*

The DPPH• scavenging activity of *R. ponticum* leaf extracts was determined according to Blois (1958). Briefly, 160 mL of 0.1 mM DPPH, prepared in MeOH, was added to 40 mL of extract solutions in MeOH at various concentrations. The absorbance of each extract was measured at 517 nm after 30 min.

2.3.3. *ABTS+• scavenging activity assay*

ABTS+• scavenging activities assay was used to determine the analysis samples (Re *et al.*, 1999). In this method, the lightening of the ABTS+• solution was measured. Before using the ABTS+• solution, it was diluted with EtOH. 160 µL of ABTS+• solution was added to 40 µL of extract solution at different concentrations in a 96-well microplate. Then, the mixture was incubated for 10 min at 20°C. The absorbance of extracts was measured at 734 nm.

2.3.4. *Cupric reducing antioxidant capacity (CUPRAC)*

The cupric-reducing antioxidant capacity of *R. ponticum* leaves extracts was determined according to the method described by Apak *et al.* (2004). To each well, 40 µL extract in various concentrations, aqueous solutions including 50 µL $CuCl_2.2H_2O$ (10 mM), 50 µL neocuproine (7.5 mM), and 60 µL NH_4Ac buffer (1 M, pH 7.0) were added. After one hour, the absorbances of the extracts were measured. BHT and α-TOC were used as antioxidant standards to compare the activity results. The results were given as $A_{0.5}$, which corresponded to the concentration versus 0.500 absorbance.

3. RESULTS

The HPLC-DAD results of acetone and methanol extracts of *R. ponticum* leaves are given in Table 1. According to the results, 14 phenolic compounds, namely, epicatechin (31.15 µg/mL), catechin (25.80 µg/mL), myricetin (8.02 µg/mL), pyrocatechol (5.77 µg/mL), rutin (5.49 µg/mL), theobromine (5.21 µg/mL), *p*-coumaric acid (4.87 µg/mL), 4-hydroxy benzaldehyde (3.08 µg/mL), fisetin (2.48 µg/mL), taxifolin (2.29 µg/mL), theophylline (1.63 µg/mL), fumaric acid (1.53 µg/mL), gallic acid (0.61 µg/mL), genistein (0.34 µg/mL) were elucidated in acetone extract. Among them, the major phenolics quantified were epicatechin (31.15 µg/mL) and catechin (25.80 µg/mL).

Table 1. Phenolic component analysis results of *R. ponticum* extracts with the HPLC-DAD (mg/g).

Phenolic compound	RT (min)	Acetone extract	Methanol extract
Fumaric acid	14.014	1.53± 0.04	-
Gallic acid	15.225	0.61±0.01	0.92±0.02
Pyrocatechol	24.658	5.77±0.14	7.69±0.19
Theobromine	25.967	5.21±0.13	-
Theophylline	29.449	1.63±0.04	-
Catechin	30.274	25.80±0.64	33.08±0.82
4-hydroxy benzaldehyde	33.367	3.08±0.08	-
Epicatechin	35.278	31.15±0.78	26.54±0.66
p-coumaric acid	40.874	4.87±0.12	2.02±0.05
Taxifolin	41.200	2.29±0.06	-
Rutin	47.527	5.49±0,14	4.44±0.11
Myricetin	50.368	8.02±0.20	6.13±0.15
Fisetin	51.243	2.48±0.06	-
Genistein	57.739	0.34±0.01	-
Luteolin	57.872	-	0.24±0.01

In methanol extract, however, eight phenolic compounds, namely, catechin (33.08 µg/mL), epicatechin (26.54 µg/mL), pyrocatechol (7.69 µg/mL), myricetin (6.13 µg/mL), rutin (4.44 µg/mL), *p*-coumaric acid (2.02 µg/mL), gallic acid (0.92 µg/mL), luteolin (0.24 µg/mL) were elucidated. Methanol extract of catechin (33.08 µg/mL) and epicatechin (26.54 µg/mL) were also quantified as major phenolics.

The antioxidant activity results of extracts are given in Table 2. The acetone extract (IC_{50}: 1.29±0.76 µg/mL) exhibited higher lipid peroxidation activity than the methanol extract. It also demonstrated higher activity than *α*-Tocopherol (IC_{50}: 4.50±0.09 µg/mL) and BHT (IC_{50}: 2.34±0.09 µg/mL). The methanol extract also indicated higher lipid peroxidation inhibitory activity (IC_{50}: 4.64±0.83 µg/mL), which is comparable with *α*-tocopherol and BHT.

The $DPPH^{•}$ scavenging activity of acetone and methanol extracts are compared with *α*-Tocopherol (IC_{50}: 12.26±0.07 µg/mL) and BHT (IC_{50}: 54.97±0.99 µg/mL) (Table 2). The acetone extract (IC_{50}: 10.21±0.56 µg/mL) exhibited excellent radical scavenging activity. On the other hand, the methanol extract (IC_{50}: 19.03±0.09 µg/mL) also demonstrated superior activity. Similarly, the $ABTS^{+•}$ scavenging activity of acetone. (IC_{50}: 2.11±0.22 µg/mL) and methanol (IC_{50}: 2.67±0.64 µg/mL) extracts indicated excellent cation radical scavenging activity. In the same conditions, *α*-Tocopherol and BHT demonstrated4.87±0.45µg/mL and IC_{50}: 2.91±0.55 µg/mL IC_{50} values. The CUPRAC assay also provided results similar to those of the $ABTS^{+•}$ and $DPPH^{•}$ assays. The acetone extract ($A_{0.5}$: 9.34±0.00 µg/mL) also exhibited higher activity than the methanol extract ($A_{0.5}$: 10.28±0.01 µg/mL). Both extracts competed with the positive standards BHT ($A_{0.5}$: 4.00±0.04 µg/mL) and α-Tocopherol ($A_{0.5}$: 25.55±0.04 µg/mL).

Table 2. Antioxidant activity of *R. ponticum* extracts.

Extract	*β*-carotene/linoleic acid assay IC_{50} (µg/mL)	$DPPH^{•}$ assay IC_{50} (µg/mL)	$ABTS^{+•}$ assay IC_{50} (µg/mL)	CUPRAC assay $A_{0.5}$ (µg/mL)
Methanol	4.64±0.83	19.03±0.09	2.67±0.64	10.28±0.01
Acetone	1.29±0.76	10.21±0.56	2.11±0.22	9.34±0.00
α-TOC*	4.50±0.09	12.26±0.07	4.87±0.45	25.55±0.04
BHT*	2.34±0.09	54.97±0.99	2.91±0.55	4.00±0.04

*α-TOC: *α*-tocopherol and *BHT: butylated hydroxytoluene were used as standards.
* Values expressed are the mean ± SEM of three parallel measurements (p<0.05).

4. DISCUSSION and CONCLUSION

In our study, the phenolic profile and antioxidant activity of the acetone and methanol extracts of the leaves of *R. ponticum* collected from Trabzon Akçaabat, Türkiye were studied. Fourteen phenolic compounds were detected in the acetone extract, while eight were in the methanol extract. Considering the phenolic contents of both extracts, catechin and epicatechin were major compounds. According to the antioxidant activity test results, acetone extract showed excellent activity, which is superior to positive standards and methanol extract in all antioxidant assays. The higher amount of phenolic compounds in acetone extract is responsible for the activity. This study also reveals that *Rhododendron ponticum* contains more amount of catechin and epicatechin. In order to make maximum use of the rich catechin and epicatechin content of *R. ponticum* in the food and pharmaceutical industries, further studies are required to optimize these compounds.

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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

Yusuf Sıcak: Investigation, Finding Materials, Extraction and Writing. **İrfan Öztürk**: Investigation, Activity experiments. **Bihter Şahin**: Interpretation of Results, Writing. **Dilaycan Çam**: Extraction, Activity Experiments. **Cansel Çakır**: Extraction, Activity Experiments. **Mehmet Öztürk**: Supervision, Interpretation of Results.

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REFERENCES

Al-Mamary, M., Al-Meeri, A., & Al-Habori, M. (2002). Antioxidant activities and total phenolics of different types of honey. *Nutrition Research*, *22*, 1041-1047.

Apak, R., Güçlü, K., Özyürek, M., & Karademir, S.E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC Method. *Journal of Agricultural and Food Chemistry, 52*, 7970-7981.

Baytop, T. (1999). *Therapy with Medicinal Plants in Turkey: Past and Present (Second edition)*. Nobel Tıp Kitabevleri.

Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*, 1199-1200.

Caliskan, H., Argon, M., & Sabudak, T. (2022). The genus Isatis L.: A review on its flavonoid and phenolic compound profile. *Records of Agricultural and Food Chemistry*, *2*(2), 75-83.

Chosson, E., Chaboud, A., Chulia, A.J., & Raynoud, J. (1998). Dihydroflavonol glycosides from *Rhododendron ferrugineum. Phytochemistry*, *49*, 1431-1433.

Erdemoglu, N., Akkol, E. K., Yesilada, E., & Calış, I. (2008). Bioassay-guided isolation of anti-inflammatory and antinociceptive principles from a folk remedy, *Rhododendron ponticum* L. leaves. *Journal of Ethnopharmacology*, *119*(1), 172-178.

Jing, X., Zhonghua, Z., Jianshe, L., & Yang, W. (2009). The effect of root of Rhododendron on the activation of NF-κ B in a chronic glomerulonephritis rat model. *Journal of Nanjing Medical University*, *23*, 73.

Li, C.-J., Wang, L.-Q., Chen, S.-N., & Qin, G.-W. (2000). Diterpenoids from the fruits of *Rhododendron molle*. *Journal of Natural Products*, *63*, 1214-1217.

Mahomoodally, M.F., Sieniawska, E., Sinan, K.I., Nancy, Picot-Allain, M.C., Yerlikaya, S., Cengiz Baloglu, M., & Zengin, G. (2019). Utilisation of *Rhododendron luteum* Sweet bioactive compounds as valuable source of enzymes inhibitors, antioxidant, and anticancer agents. *Food and Chemical Toxicology*, 111052.

Miller, H.M. (1971). A simplified method for the evaluation of antioxidants. *Journal of the American Oil Chemists Society,* 48, 91.

Oztasan, N., Altinkaynak, K., Akcay, F., Gocer, F., & Dane, S. (2005). Effect of mad honey on blood glucose and lipid levels in rats with streptozocin-induced diabetes. *Turkish Journal of Veterinary and Animal Science*, *29*, 1093-1096.

Prakash, T.F., Snehal, D.S., Uday R.S., & Diyakar. (2008). Hepatoprotective activity of leaves of *Rhododendron arboreum* in CCl_4 induced hepatotoxicity in rats. *Journal of Medical Research*, *2*(11), 315-320.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*, 1231-1237.

Robards, K., Prenzler, P.D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry*, *66*, 401-436.

Sales, F., & Milne, R. Güner, A., Özhatay, N., Ekim, T., & Başer, K.H.C. (Eds.) (2000). Rhododendron L. *Flora of Turkey and the East Aegean Islands*, University Press, Edinburgh, 11, 181-183.

Shrestha, A., Hakeem Said, I., Grimbs, A., Thielen, N., Lansing, L., Schepker, H., & Kuhnert, N. (2017). Determination of hydroxycinnamic acids present in *Rhododendron* species. *Phytochemistry*, 144, 216-225.

Silici, S., Sagdic O., & Ekici, L. (2010). Total phenolic content, antiradical, antioxidant, and antimicrobial activities of *Rhododendron* honeys. *Food Chemistry*, *121*(1), 238-243.

Stevens, P., & Fernandez, J.W. (1978). Fang Architectonics. *African Arts*, *11*(2), 90-92. https://doi.org/10.2307/3335466

Tokul Ölmez, Ö., Şahin, B., Çakır, C., & Öztürk, M. (2020). Rapid and easy method for simultaneous measurement of widespread 27 compounds in natural products and foods. *Journal of Chemical Metrology*, *14*(1), 1-11.

Yarlioglues, M., Akpek, M., Ardic, I., Elcik, D., Sahin, O., & Kaya, M.G. (2011). Mad-honey sexual activity and acute interior myocardial infarctions in a married couple. *Texas Heart Institute Journal*, *38*, 577-580.

Yeşil, T., & Akgül, Y. (2022). Major components of *Rhododendron luteum* leaves. *Natural Product Research*, *37*(15), 2608-2612. https://doi.org/10.1080/14786419.2022.2055015

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Research Article

Effective inhibition of bacterial sialidases by phenolic acids and flavonoids

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Abstract: As a pathogenicity factor in some microorganisms, sialidase is a key target for inactivation, as this would have curative and preventive effects on various diseases. Significant results are already achieved with viral sialidase inhibitors, while such studies on bacterial enzymes are scarce. Pure natural compounds representing phenols and flavonoids, were tested for their inhibitory effect on sialidases from *Vibrio cholerae* non-O1, *Arthrobacter nicotianae* and *Oerskovia paurometabola*. All three enzymes were isolated, purified beforehand and stored under suitable conditions. Quinic and gallic acids showed the highest inhibitory activity - 76 to 100% against the three sialidases. Fisetin had a significant inhibitory activity on two of the enzymes. The structurally related thymol and thymoquinone exerted from 80 to 100% inhibition on at least one of the enzymes. Catechin and rutin had significant inhibitory activity, varying from 49 to 100%, on some of the enzymes. Quercetin, known for its inhibitory effect on viral sialidases, had a lesser impact on the studied enzymes. The suppressive effect of quinic acid, rutin and fisetin on bacterial sialidases is observed for the first time.

1. INTRODUCTION

Sialidases are glycoside hydrolases (EC 3.2.1.18, exo-alpha sialidases, neuraminidases) that are present in the metabolism of humans and animals of the Deuterostomata lineage, and in some of their parasitic or commensal microorganisms such as viruses, bacteria, fungi, unicellular eukaryotes (Giacopuzzi *et al*., 2012). These enzymes cleave terminal sialic acids from complex sialosides including glycoproteins, glycolipids, polysaccharides, and polysialic compounds. As a result, free sialic acid is released (Schauer & Kamerling, 2018) (Figure 1). Sialic acids are a class of alpha-keto acid sugars with a nine-carbon backbone. The most common member of this group is N-acetylneuraminic acid (Neu5Ac) found in animals, humans, and some prokaryotes.

Some of the best-studied microbial sialidases are the viral ones, known so far in the families *Orthomyxoviridae* (for influenza viruses of type A and B) and *Paramyxoviridae*. They are a factor of pathogenicity, facilitating the adhesion and penetration of virus particles into host cells, as well as the spread of the newly formed virions. This enzyme is also a key factor in the pathogenesis of a number of bacterial infections, such as cholera, gas gangrene, meningitis,

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septicemia, cystic fibrosis, etc. (Corfield, 1992; Brittan *et al.*, 2012). By cleaving terminal sialic residues from mucins, sialidase disrupts the mucosal layer, thereby allowing the infectious agent to penetrate to the tissue surface. By removing sialic residues from glycoproteins located on the cell surface, the enzyme reveals receptors to which the microorganisms adhere.

Figure 1. Schematic presentation of sialidase action.

The sialic acid derivative 2-deoxy-2, 3-didehydro-D-N-acetylneuraminic acid (Neu5Ac2en, DANA) was the first sialidase inhibitor, which mimics the oxocarbenium ion-like transition state (Meindl *et al.*, 1974). DANA inhibits most sialidases and is a key compound in the search for antiviral drugs, leading to the synthesis of the highly effective commercial products Zanamivir™ and oseltamivir (Tamiflu™). At the same time, emergence of strains that are resistant even to these effective antiviral agents is reported (Grienke *et al.*, 2012). Several triazole-linked derivatives of DANA were synthesized as selective inhibitors against *Vibrio cholerae* and *Arthrobacter ureafaciens* sialidases (Slack *et al.*, 2018).

Since the discovery of the effective inhibitors of influenza virus sialidase and especially after the emergence of influenza strains resistant to them, the search for novel inhibitors is ongoing. It also develops in the direction of bacterial sialidases, where data are so far scarce. In recent years, a number of secondary plant metabolites have emerged as reliable sources of potential sialidase inhibitors (Sadati *et al.*, 2019). Phenolic compounds are secondary metabolites including simple phenols, phenolic acids, flavonoids, xanthones, coumarins, stilbenes, tannins, lignans (Vuolo *et al.*, 2019). A wide range of biological activities including antimicrobial, antioxidant, anti-inflammatory, antidiabetic, neuroprotective, and hepatoprotective have been reported for them. The health effect of these compounds is determined in many cases by their ability to inhibit enzymes associated with various human diseases like hypertension, metabolic problems, incendiary infections, and neurodegenerative diseases (Gonçalves and Romano, 2017; Rahman *et al.*, 2021).

We have chosen for our study a set of substances, which include a precursor of oseltamivir (quinic acid), structurally related to it gallic acid; compounds with studied sialidase inhibition properties (catechin, quercetin), and ones that are not studied in this aspect (rutin, fisetin, thymol and thymoquinone).

2. MATERIAL and METHODS

2.1. Bacterial Sialidases

Sialidases from three bacterial producer strains were used: *Arthrobacter nicotianae* (AN), *Oerskovia paurometabola* Strain O129 (NBIMCC 9093) (O129), and *Vibrio cholerae* non-O1 Strain V13 (NBIMCC 8716) (V13). The enzymes were purified earlier and stored as lyophilized powder for AN (Abrashev *et al.*, 2005), or as phosphate buffered saline (PBS) solution kept at -20 ºC for O129 and V13 (Eneva *et al.*, 2015; Eneva *et al.*, 2022). Before performing the inhibition test, the enzymes were standardized to solutions with 10 U/mL sialidase activity by dilution in PBS.

2.2. Chemical Compounds

In the present study, flavonoid aglycones: quercetin (3,3′,4′,5,7-pentahydroxyflavone), fisetin (3,3′,4′,7-tetrahydroxyflavone), catechin (3,3′,4′,5,7-pentahydroxyflavane) and flavonoid glycoside rutin (quercetin 3-rutinoside,) were selected for evaluation of their sialidase

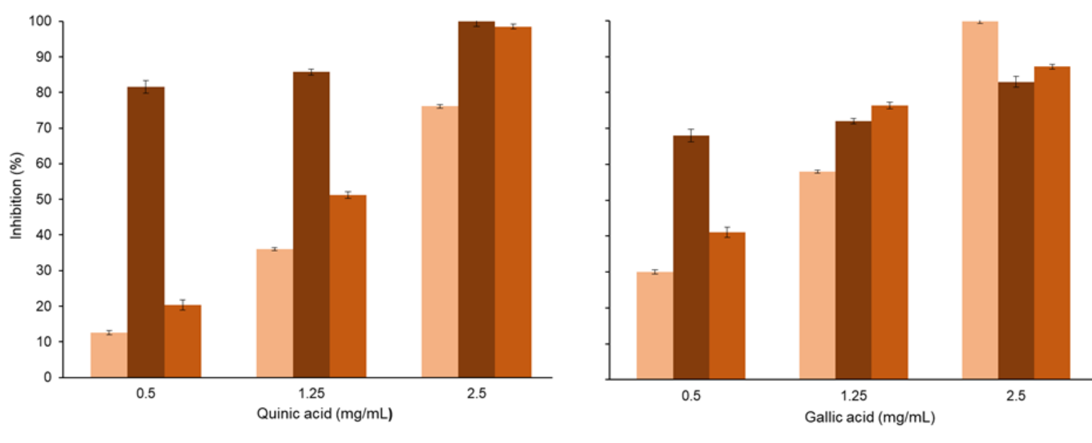
inhibitory activity. Gallic acid, ellagic acid and quinic acid from the phenolic acids and thymol (2-isopropyl-5-methylphenol), and thymoquinone (2-isopropyl-5-methyl-1, 4-benzoquinone) as monoterpenoid phenols were assayed too. Thymol, thymoquinone, gallic acid, ellagic acid, quinic acid, fisetin, rutin, catechin, and quercetin were obtained from Sigma–Aldrich.

2.3. Enzyme Assay

Sialidase activity was assayed according to the thiobarbituric acid method of Uchida *et al.* (1977). Glucomacropeptide (GMP) obtained from cheese whey was used as a substrate (Abrashev *et al.*, 1980). Separate 200-µl enzyme samples were mixed with 200 µl of each of the tested compounds dissolved in 5% dimethyl sulfoxide (DMSO). Control 200-µl enzyme samples mixed with 200 µl 5% DMSO in PBS were also prepared. Samples of each incubation mixture were assayed and the absorbance of samples and controls was measured on a UV-VIS 75 at λ=551nm. The amount of sialic acids released was determined as the difference (ΔE) in the extinctions of the sample and control was plotted on a standard curve, created using sialic acid as a standard. One unit of sialidase activity is defined as the amount that releases 1 µmol of N-acetylneuraminic acid (Neu5Ac) for 1 min at 37 °C using GMP as a substrate. Results are displayed as relative activity compared to the control, which was set as 100% activity. All experiments were performed in triplicate, and the data is reported as the average of three sample replicates, accompanied by the standard deviation.

3. RESULTS and DISCUSSION

Since the enzymes used in this study and most of the tested substances are being studied for the first time, we applied relatively high concentrations of all components (0.5, 1.25, 2.5 mg/mL). Our results show that quinic and gallic acids have the highest inhibitory activity. With the three sialidases tested, they exert inhibition in the range of 76-100% for the concentration of 2.5 mg/mL. AN and V13 sialidases are weakly inhibited with decreasing the acid concentration, while O129 enzyme retains approximately the same levels of high inhibition independently from the decreasing acid concentration (Figure 2). The inhibition effect is expected given the fact that quinic acid is a starting substance for the synthesis of oseltamivir, and gallic acid is close to it in structure. Quinic acid is a cyclic polyol, found in the bark of *Cinchona* trees, in the coffee beans, and also in *Urtica dioica*. In plants, quinic acid and compounds similar to it are precursors to lignins and phenols and they accumulate to some extent, especially in gymnosperms and woody dicotyledons (Farina and Brown, 2006).



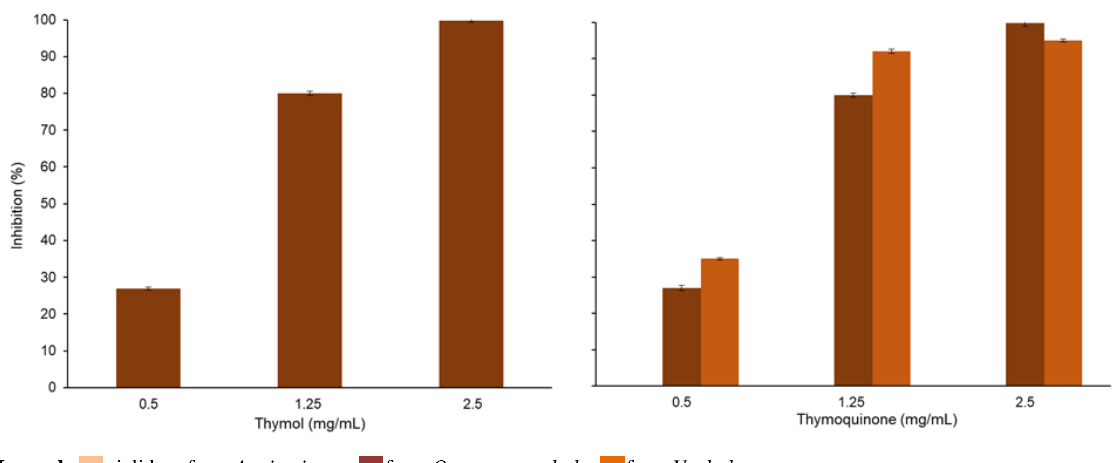
Legend: sialidase from *A. nicotianae*, from *O. paurometabola*, from *V. cholerae*

Figure 2. Inhibition effects of quinic and gallic acid on three bacterial sialidases

To our knowledge, the results we obtained are the first to describe the direct inhibitory effect of quinic acid on bacterial sialidase. It is known for its significantly suppressive effect on influenza A and PPR virus sialidases (Gattani *et al.*, 2020). Gallic acid is found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants (Haslam and Cai, 1994). They

represent a large family of plant secondary metabolites which is found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants. Gallic acid has antioxidant, antifungal, antimicrobial, and anticancer activities (Haslam and Cai, 1994; Rosas *et al*., 2019).

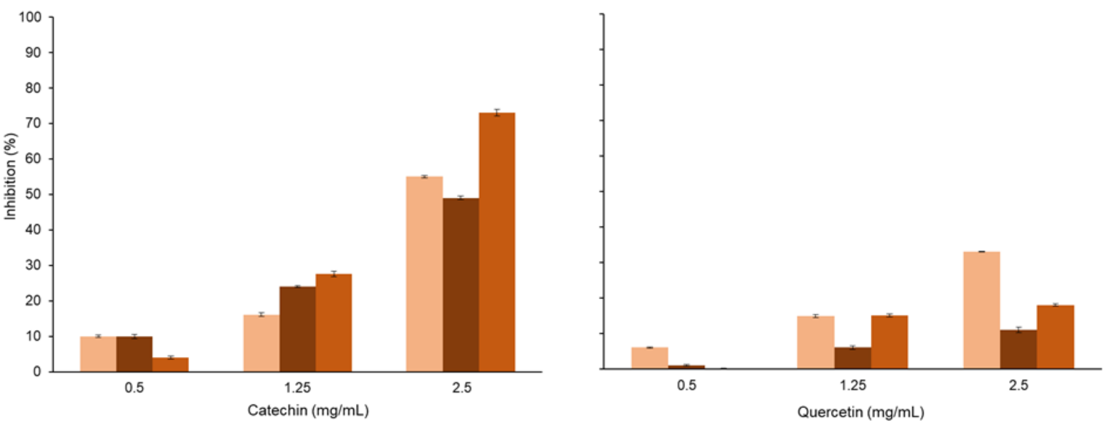
The monoterpenes thymol and thymoquinone have a similar strong effect, but with two of the sialidases. When tested in concentration 2.5 mg/mL, thymol has practically 100% inhibitory effect on only one of the enzymes - that of O129, and thymoquinone - on the enzyme of O129 and V13 (Figure 3). Thymol is a monoterpenoid phenol that is contained in thyme and other plants such as *Ocimum gratissimum* L., *Origanum* L., *Carum copticum* L., different species of the genus *Satureja* L., *Oliveria decumbens* Vent, etc., and has strong antiseptic properties. Thymol has shown an inhibitory effect on biofilms *in vitro* (Escobar *et al*., 2020). Both thymol and thyme essential oil were proved to have antibiofilm, antifungal, antileishmanial, antiviral, and anticancer properties (Kowalczyk *et al*., 2020). Thymoquinone is found in many plants of the Lamiaceae family, such as *Origanum*, *Monarda*, *Satureja*, *Thymus*, but in a particularly high concentration was found in *Nigella sativa* (Farkhondeh *et al*., 2017). However, it belongs to the group of substances that interfere with many qualitative reactions, as it reacts non-specifically with a large number of substances of interest (Baell, 2016), therefore, the results of this trial should be interpreted with caution.



Legend: sialidase from *A. nicotianae*, from *O. paurometabola*, from *V. cholerae*

Figure 3. Inhibition effects of thymol and thymoquinone on three bacterial sialidases.

The tested substances from the flavonol group inhibit the three sialidases to varying degrees. Catechin, at the highest tested concentration (2.5 mg/mL) displays strong suppressing activity - from 49 to 73% for all three enzymes (Figure 4). Some catechins were proved to interact with influenza neuraminidase. Moreover, these compounds bind to a site different from that to which known inhibitors like zanamivir and oseltamivir bind, in the vicinity of a structurally conserved cavity adjacent to residue 430 that has been suggested to be a secondary sialic acid binding site, thereby overcoming mutations limiting influenza therapy (Mueller and Downard, 2015). In addition to influenza virus neuraminidase, epigallocatechin-3-gallate successfully suppresses *Clostridium perfringens* neuraminidase (Li *et al*., 2011; Kim *et al*., 2013). Catechins are a class of flavonoids, secondary metabolites in plants distributed in a variety of foods and herbs including tea, apples, persimmons, cacaos, grapes, and berries and is known for its anticancer, anti-obesity, antidiabetic, anticardiovascular, anti-infectious, hepatoprotective, and neuroprotective properties (Isemura, 2019). Regarding the bacterial sialidases tested by us, no significant inhibition of enzyme activity by quercetin was observed. Quercetin has a variety of pharmacological properties including anti-SARS-CoV-2, antioxidant, anticancer, antiaging, antiviral, and anti-inflammatory activities (Wang *et al*., 2022). A recent study revealed that O-methylated quercetin derivatives from the aerial parts of *Siegebeckia pubescens* have inhibitory effect on bacterial sialidase (Son *et al*., 2023). According to Sadati *et al*. (2019), quercetin and catechin, along with other flavonoids such as naringenin, luteolin, hispidulin, vitexin, chrysin and kaempferol may effectively block the sialidase active site.

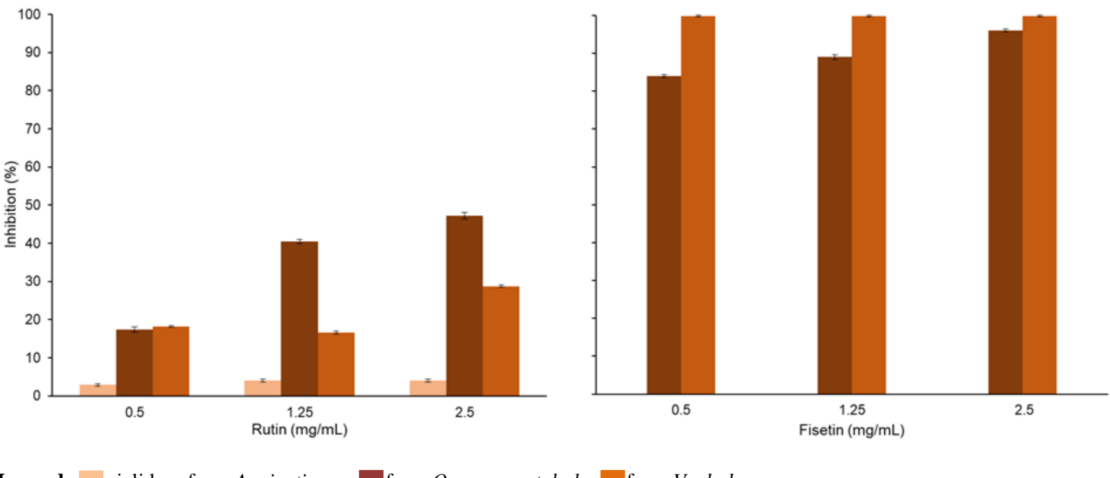


Legend: sialidase from *A. nicotianae*, from *O. paurometabola*, from *V. cholerae*

Figure 4. Inhibition effects of catechin and quercetin on three bacterial sialidases.

A recent study on influenza virus NA revealed that the presence of a glycosylation group greatly reduces NA inhibition (Chon, 2012). In our study, the glycosylated flavonol rutin had the best inhibitory effect only on sialidase from O129 – 49% inhibition (Figure 5). Studies that have tested this flavonol for inhibition of viral sialidases report its action as moderate to weak (Liu *et al*., 2008, Mercader and Pomilio, 2010). Rutin is widespread in plants, such as passion flower, buckwheat, tea, and apple. It has a number of pharmacological activities, including anticarcinogenic, antioxidant, antiviral, vasoprotective and hepatoprotective activities (Ganeshpurkar and Saluja, 2017). There is no evidence of suppressive effect of rutin on bacterial sialidases in the literature till now. This compound is described to exhibit a significant inhibitory effect on α-amylase and α-glucosidase - enzymes with a related mechanism of action to that of sialidase (Dubey *et al*., 2017).

In contrast to rutin, for two of the sialidases, produced by O129 and V13, the inhibitory effect of the plant flavonol fisetin was comparable and even higher than that of quinic and gallic acids, 96 and 100%, respectively. As could be seen in Figure 5, fisetin significantly decreases the effectiveness (about 96%) of O129 and V13 sialidases even at the lowest tested concentration (0.5 mg/mL). Further studies are required to establish the minimum concentration of fisetin that has significant inhibitory effect on these enzymes. Fisetin is present in several fruits and vegetables including strawberries, apples, persimmons, onions and has antioxidant, anti-inflammatory and anticancer activity (Sahu *et al*., 2014). There are no data in the available literature regarding inhibitory activity of fisetin towards sialidase.



Legend: sialidase from *A. nicotianae*, from *O. paurometabola*, from *V. cholerae*

Figure 5. Inhibition effects of rutin and fisetin on tree bacterial sialidases.

4. CONCLUSION

Inhibition of bacterial sialidase activity is a practical approach for the treatment of different microbial infections. In our study, quinic and gallic acids, close to the compounds from which oseltamivir is produced, were tested and found to significantly suppress all the three enzymes examined. Fisetin is of interest for additional research due to its strong inhibition of *V. cholerae* and *O. paurometabola* sialidases, even at the lowest concentration tested. The specific effect that most of the tested substances gave on each of the enzymes indicates that specific inhibitors for individual bacterial sialidases can be sought among such natural products. Future studies on the still poor explored kinetics of bacterial sialidases inhibition by pure phenolic and flavonoid compounds will provide useful information on their potential application as antibacterial and therapeutic substances of natural origin. Our findings prove that the tested substances are able to inhibit enzymes that represent factors of pathogenicity.

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

Yana Gocheva: Conception, Design, Analysis, Literature Review, Writing, Critical Review. **Milena Nikolova**: Materials, Literature Review, Writing. **Stephan Engibarov**: Data Collection, Analysis, Literature Review, Writing, Critical Review. **Irina Lazarkevich:** Literature Review, Writing. **Rumyana Eneva:** Supervision, Analysis and Interpretation, Writing.

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REFERENCES

Abrashev, I., Velcheva, P., Nikolov, P., & Kourteva, J. (1980). Substrate for colorimetric determination of enzyme activity. Bulgarian patent N 47647/IIR

Abrashev, I., Dulguerova, G., Dolashka-Angelova, P., & Voelter, W. (2005). Purification and Characterization of a Novel Sialidase from a Strain of *Arthrobacter nicotianae*. *Journal of Biochemistry*, *137*(3), 365-371. https://doi.org/10.1093/jb/mvi053

Baell, J.B. (2016). Feeling nature's PAINS: natural products, natural product drugs, and pan assay interference compounds (PAINS). *Journal of Natural Products*, *79*(3), 616-628. https://doi.org/10.1021/acs.jnatprod.5b00947

Brittan, J.L., Buckeridge, T.J., Finn, A., Kadioglu, A., & Jenkinson, H.F. (2012). Pneumococcal neuraminidase A: an essential upper airway colonization factor for *Streptococcus pneumoniae*. *Molecular Oral Microbiology*, *27*(4), 270-283. https://doi.org/10.1111/j.2041-1014.2012.00658.x

Chon, H. (2012). *Medicinal Herbs and Plant Extracts for Influenza: Bioactivity, Mechanism of Anti-influenza Effects, and Modulation of Immune Responses.* Studies in Natural Products Chemistry, Elsevier, https://doi.org/10.1016/B978-0-444-59530-0.00011-3

Corfield, T. (1992). Bacterial sialidases - roles in pathogenicity and nutrition. G*lycobiology*, *2*(6), 509-521. https://doi.org/10.1093/glycob/2.6.509

Dubey, S., Ganeshpurkar, A., Ganeshpurkar, A., Bansal, D., & Dubey, N. (2017). Glycolytic enzyme inhibitory and antiglycation potential of rutin. *Future Journal of Pharmaceutical Sciences*, *3*(2), 158-162. https://doi.org/10.1016/j.fjps.2017.05.005

Eneva, R., Engibarov, A., Petrova, P., Abrashev, R., Strateva, T., Kolyovska, V., & Abrashev, I. (2015). High production of neuraminidase by a *Vibrio cholerae* non-O1 strain-the first possible alternative to toxigenic producers. *Applied Biochemistry and Biotechnology*, *176*, 412-427. https://doi.org/10.1007/s12010-015-1584-4

Eneva, R., Engibarov, S., Gocheva, Y., Mitova, S., Arsov, A., Petrov, K., Abrashev, R., Lazarkevich, I., & Petrova, P. (2022). Safe sialidase production by the saprophyte *Oerskovia paurometabola*: Gene sequence and enzyme purification. *Molecules*, *27*(24), 8922. https://doi.org/10.3390/molecules27248922

Escobar, A., Perez, M., Romanelli, G., & Blustein, G. (2020). Thymol bioactivity: A review focusing on practical applications. *Arabian Journal of Chemistry*, *13*(12), 9243-9269. https://doi.org/10.1016/j.arabjc.2020.11.009

Farina, V., & Brown, J.D. (2006), Tamiflu: The Supply Problem. *Angewandte Chemie International Edition*, *45*(44) 7330-7334. https://doi.org/10.1002/anie.200602623

Farkhondeh, T., Samarghandian, S., & Borji, A. (2017). An overview on cardioprotective and anti-diabetic effects of thymoquinone. *Asian Pacific Journal of Tropical Medicine*, *10*(9), 849-854. https://doi.org/10.1016/j.apjtm.2017.08.020

Ganeshpurkar, A., & Saluja, A.K. (2017). The pharmacological potential of rutin. *Saudi Pharmaceutical Journal*, *25*(2), 149-164. https://doi.org/10.1016/j.jsps.2016.04.025

Gattani, A., Kumar, A., Agrawal, A., Khan, M.H., Mahawar, M., Bag, S., Rajak, K.K., Singh, R.K., & Singh, P. (2020). An electrochemical impedance sensor for monitoring of gallic acid inhibited neuraminidase activity of PPR HN protein. *Microchemical Journal*, *158*, 105229. https://doi.org/10.1016/j.microc.2020.105229

Giacopuzzi, E., Bresciani, R., Schauer, R., Monti, E., Borsani, G. (2012) New insights on the sialidase protein family revealed by a phylogenetic analysis in Metazoa. *PLoS One*, *7*(8), e44193. https://doi.org/10.1371/journal.pone.0044193

Gonçalves, S., & Romano, A. (2017). Inhibitory properties of phenolic compounds against enzymes linked with human diseases. *Phenolic Compounds-biological Activity*, INTECH, http://dx.doi.org/10.5772/66844

Grienke, U., Schmidtke, M., von Grafenstein, S., Kirchmair, J., Liedl, K.R., & Rollinger, J.M. (2012). Influenza neuraminidase: a druggable target for natural products. *Natural Product Reports*, 29(1), 11-36. https://doi.org/10.1039/C1NP00053E

Haslam, E., & Cai, Y. (1994). Plant polyphenols (vegetable tannins): gallic acid metabolism. *Natural Product Reports*, *11*, 41-66. https://doi.org/10.1039/NP9941100041

Isemura, M. (2019). Catechin in human health and disease. *Molecules*, *24*(3), 528. https://doi.org/10.3390/molecules24030528

Kim, M., Kim, S.Y., Lee, H.W., Shin, J.S., Kim, P., Jung, Y.S., ... & Lee, C.K. (2013). Inhibition of influenza virus internalization by (−)-epigallocatechin-3-gallate. *Antiviral Research*, *100*(2), 460-472. https://doi.org/10.1016/j.antiviral.2013.08.002

Kowalczyk, A., Przychodna, M., Sopata, S., Bodalska, A., & Fecka, I. (2020). Thymol and thyme essential oil—new insights into selected therapeutic applications. *Molecules*, *25*(18), 4125. https://doi.org/10.3390/molecules25184125

Li, J., Sayeed, S., Robertson, S., Chen, J., McClane, B.A. (2011) Sialidases affect the host cell adherence and epsilon toxin-induced cytotoxicity of *Clostridium perfringens* type D strain CN3718. *PLoS Pathogenes, 7*(12), e1002429. https://doi.org/10.1371/journal.ppat.1002429

Liu, A.L., Wang, H.D., Lee, S.M., Wang, Y.T., & Du, G.H. (2008). Structure–activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities. *Bioorganic & Medicinal Chemistry*, *16*(15), 7141-7147. https://doi.org/10.1016/j.bmc.2008.06.049

Meindl, P., Bodo, G., Palese, P., Schulman, J., & Tuppy, H. (1974). Inhibition of neuraminidase activity by derivatives of 2-deoxy-2, 3-dehydro-N-acetylneuraminic acid. *Virology*, *58*(2), 457-463. https://doi.org/10.1016/0042-6822(74)90080-4

Mercader, A.G., & Pomilio, A.B. (2010). QSAR study of flavonoids and biflavonoids as influenza H1N1 virus neuraminidase inhibitors. *European Journal of Medicinal Chemistry, 45*(5), 1724-1730. https://doi.org/10.1016/j.ejmech.2010.01.005

Mueller, P., & Downard, K.M. (2015). Catechin inhibition of influenza neuraminidase and its molecular basis with mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *111*, 222-230. https://doi.org/10.1016/j.jpba.2015.03.014

Rahman, M.M., Rahaman, M.S., Islam, M.R., Rahman, F., Mithi, F.M., Alqahtani, T., Almikhlafi, Alghamdi, S.Q., Alruwaili, A.S., Hossain, M.S., Ahmed, M., Das, R., Emran, T.B., & Uddin, M.S. (2021). Role of phenolic compounds in human disease: current knowledge and future prospects. *Molecules*, *27*(1), 233. https://doi.org/10.3390/molecules27010233

Rosas, E.C., Correa, L.B., & das Graças Henriques, M. (2019). Antiinflammatory properties of *Schinus terebinthifolius* and its use in arthritic conditions. *In Bioactive food as dietary interventions for arthritis and related inflammatory diseases*. Academic Press. https://doi.org/10.1016/B978-0-12-813820-5.00028-3

Sadati, S.M., Gheibi, N., Ranjbar, S., & Hashemzadeh, M.S. (2019). Docking study of flavonoid derivatives as potent inhibitors of influenza H1N1 virus neuraminidase. *Biomedical Reports*, *10*(1), 33-38. https://doi.org/10.3892/br.2018.1173

Sahu, B.D., Kalvala, A.K., Koneru, M., Mahesh Kumar, J., Kuncha, M., Rachamalla, S.S., & Sistla, R. (2014). Ameliorative effect of fisetin on cisplatin-induced nephrotoxicity in rats via modulation of NF-κB activation and antioxidant defence. *PloS one*, *9*(9), e105070. https://doi.org/10.1371/journal.pone.0105070

Schauer, R., Kamerling, J.P. (2018), *Exploration of the Sialic Acid Advances in Carbohydrate Chemistry and Biochemistry*, Academic Press; https://doi.org/10.1016/bs.accb.2018.09.001

Slack, T.J., Li, W., Shi, D., McArthur, J.B., Zhao, G., Li, Y., Xiao, A., Khedri, Z., Yu, H., Liu, Y., & Chen, X. (2018). Triazole-linked transition state analogs as selective inhibitors against *V. cholerae* sialidase. *Bioorganic & Medicinal Chemistry*, *26*(21), 5751-5757. https://doi.org/10.1016/j.bmc.2018.10.028

Son, Y.G., Kim, J.Y., Park, J.Y., Kim, K.D., Park, K.H., & Kim, J.Y. (2023). Inhibitory Potential of Quercetin Derivatives Isolated from the Aerial Parts of *Siegesbeckia pubescens* Makino against Bacterial Neuraminidase. *Molecules*, *28*(14), 5365. https://doi.org/10.3390/molecules28145365

Uchida, Y., Tsukada, Y., & Sugimori, T. (1977). Distribution of neuraminidase in *Arthrobacter* and its purification by affinity chromatography. *The Journal of Biochemistry*, *82*(5), 1425-1433.

Vuolo, M.M., Lima, V.S., & Maróstica Junior, M.R. (2019). *Phenolic Compounds: Structure, classification, and antioxidant power. In Bioactive Compounds*. Woodhead Publishing. https://doi.org/10.1016/B978-0-12-814774-0.00002-5

Wang, G., Wang, Y., Yao, L., Gu, W., Zhao, S., Shen, Z., Lin, Z., Liu, W., & Yan, T. (2022). Pharmacological activity of quercetin: an updated review. *Evidence-Based Complementary and Alternative Medicine,* Article ID 3997190. https://doi.org/10.1155/2022/3997190

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Chemical composition and potent antibacterial activities of colony-forming cyanobacteria, *Desmonostoc muscorum* (Nostocales, Cyanophyceae)

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Abstract: Cyanobacteria are important natural sources of biomolecules and active compounds with promising biological activities against a wide range of microbial pathogens. The study aimed to evaluate the chemical composition and antibacterial activities of colony-forming cyanobacteria, *Desmonostoc muscorum*. Proximate analysis showed that *D. muscorum* biomass possesses high concentration of carbohydrates (35.50 ± 0.12%), protein (20.19 ± 0.03%), and ash (16.90 ± 0.02%). The elemental composition of *D. muscorum* biomass is in a decreasing order of Ca > Mn > Mg > K > Na > Fe> Zn > Cr > Pb > Cu > Cd. Also, *D. muscorum* extract exhibited potent antibacterial activities against *Staphylococcus saprophyticus*, Methicillin-Resistant *Staphylococcus aureus*, and *Listeria monocytogenes* with MIC values of 125 µg/mL, 125 µg/mL, and 250 µg/mL, respectively. The current study documents the promising use of *D. muscorum* as good sources of microelements and compounds which can be harness for food and medical applications.

1. INTRODUCTION

An increasing number of reported cases of disease (caused by drug-resistant microorganisms) causes a significant risk to public health. Among microbial pathogens, viruses and bacteria cause 37– 70% of diseases while protozoa, fungi and helminths cause 10–30% of diseases, resulting in several deaths per year (Hirata *et al.,* 1996; Ganesan *et al.,* 2017). Recently, bacterial infections are becoming resistant to several antibiotics causing a foremost worldwide healthcare problem. For instance, *Staphylococcus aureus,* a pathogenic bacterial strain responsible for several human infections, has gained resistance to majority of clinically important antibiotics. Clinicians reported cases of hospital acquired (nosocomial) drug resistant strains of *S. aureus,* which also contains resistance to a wide array of antibiotics (Kumar *et al.,* 2010). Thus, there is a high demand to continuously discover novel bioactive compounds. Regardless of all the efforts to synthesize chemically active compounds, natural environmental samples are still the best source of new compounds (Hirata *et al.,* 1996 Kumar *et al.,* 2010; Salehghamari & Najafi, 2016; Żymańczyk-Duda *et. al.,* 2022).

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Microorganisms are considered one of the significant sources of bioactive compounds with strong antimicrobial activity over a wide array of bacterial pathogens. In general, microorganisms produce biologically active molecules that are non-essential for their growth but beneficial in defense mechanism (Ganesan *et al.,* 2017; Senhorinho *et al.,* 2018; Żymańczyk-Duda *et. al.,* 2022). Cyanobacteria are source of several active substances that have diverse applications and clinical effects in human medicine. In fact, several naturally derived antibiotics were extracted from this group of bacteria. In addition, cyanobacteria are efficient photosynthetic microorganisms that collect sunlight and convert it to useful biomass (majority of which is carbohydrate, protein, and lipids) which can be harnessed for food and industrial application (Tibbets *et al.,* 2015 Little *et al.,* 2021; Żymańczyk-Duda *et. al.,* 2022). These organisms are found in several ecological habitats such as water, soil, lake, and other marine environment. Different types of cyanobacteria are recognized for generating both internal and external compounds that possess a wide range of biological activities against bacteria, fungi, and other viruses. The production of these antimicrobial agents is influenced significantly by factors such as the temperature and pH during incubation, the duration of incubation, the components of the medium, and the intensity of light (Orhan *et al.,* 2003; Katircioglu *et al.,* 2005). Although cyanobacteria have been the subject of extensive research for bioactive compound screening by medical companies for almost 50-60 years, only a minimal portion of the cyanobacterial taxa has been reported (Orhan *et al.,* 2003; Shaieb *et al.,* 2014 Salehghamari & Najafi, 2016; Senhorinho *et al.,* 2018). Cyanobacteria such as *Microcoleous lacustris* are being used as good source of abietane diterpenes with potent antibacterial activities against *S. aureus, S. epidermidis, Salmonella typhi, Vibrio cholerae, Bacillus cereus, Bacillus subtilis, Escherichia coli,* and *Klebsiella pneumonia*. (Cock & Cheesman, 2023). In addition, *Fischerella ambigua*, *Anabaena basta, Nostoc commune,* and *Spirulina platensis* produce medically important compounds such as ambiguine isonitriles, bastadin, comnostins A-E, and calcium spirulan which are being used to treat infection caused by *Bacillus anthracis* and *Mycobacterium tuberculosis* (Katircioglu *et al.,* 2005; Cock & Cheesman, 2023). Despite these advancements, further investigations are essential to identify and evaluate the therapeutic potential of several bioactive compounds derived from cyanobacteria. Antimicrobial substances from cyanobacteria need more research that will target the safety of these compounds in antimicrobial chemotherapy, given that certain cyanobacterial species produce environmental toxins (Orhan *et al.,* 2003; Little *et al.,* 2021; Cock & Cheesman, 2023). Nevertheless, many of these compounds display relatively low toxicity, making them promising candidates for drug development. Notably, some heterocyclic and linear peptides, as well as depsipeptides, have exhibited potent activity and favorable safety profiles, prompting their development as antimicrobial chemotherapies (Katircioglu *et al.,* 2005; Żymańczyk-Duda *et. al.,* 2022; Cock & Cheesman, 2023).

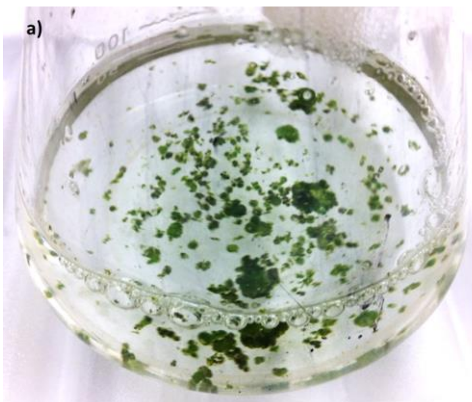
Desmonostoc muscorum is a colony-forming, filamentous nitrogen-fixing cyanobacterium capable of exhibiting wide array of physiological growth properties and cell life cycle (developmental stages) alternatives. This cyanobacterium is currently being studied for medical application due to its potent antibacterial and antioxidant properties (El-Sheekh, *et al.,* 2006; Yasin *et al.,* 2019). However, studies on the biological activities of this cyanobacterium are still limited, particularly the chemical and elemental composition as well as the antimicrobial properties of the cyanobacteria. Thus, the study aimed to evaluate the chemical composition and antibacterial activities of *Desmonostoc muscorum* BIOTECH 4087 isolated from rice paddies in Laguna, Philippines.

2. MATERIAL and METHODS

2.1. Microalgal Culture and Mass Production

The pure culture of cyanobacterium, *Desmonostoc muscorum* BIOTECH 4087 was obtained from the Philippine National Collection of Microorganisms (PNCM), National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños

(UPLB) (Figure 1). Briefly, 100 mL of *Desmonostoc muscorum* culture was grown into three 1 L flasks containing BG 11 medium (Arguelles, 2022; Arguelles, 2023). The culture media used in this study was sterilized using autoclave at 15 psi for 15 min. Mass production of *D. muscorum* was done for 24 days under 12:12 light condition (light intensity = 120 µmol photons m^{-2} s^{-1}) and kept at 23 ± 2 °C. The culture set up was bubbled continuously with filtered air from an air pump (gas velocity = 300 mL min^{-1}). The biomass of *D. muscorum* was collected using centrifugation (10,000 rpm for 10 min). The collected biomass was freeze-dried via Virtis Freeze mobile 25 SL lyophilizer to generate dried cyanobacterial biomass (Arguelles, 2023).



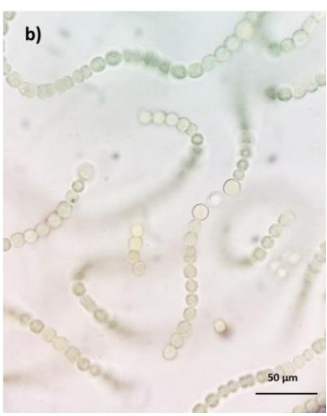


Figure 1. *Desmonostoc muscorum* BIOTECH 4087 in cultured flask (a) and a photomicrograph of the heterocystous filaments (b).

2.2. Cyanobacterial Extract Preparation

The algal extract was prepared by subjecting 1 gram of freezed-dried biomass of *D. muscorum* in 20 mL of methanol placed in an ultrasonic bath with continuous stirring for 1 hr. The extraction mixtures were centrifuged for 10 min at 12,000 rpm with a temperature set at 4 °C. Following extraction, the crude algal extracts underwent a decolorization process employing activated charcoal bleaching to remove pigments in the extract. Briefly, 1 mg/mL of activated charcoal powder was applied to the crude extract. Following the addition of activated charcoal and vortexing for 1 minute, the mixture underwent centrifugation for 5 minutes at 2,000 g, and the resulting supernatant was filtered through a polytetrafluoroethylene (PTFE) (0.22 µm) membrane. The cyanobacterial extracts were further concentrated via a rotary evaporator and were kept at 4°C (refrigerated condition) before use in the different antibacterial assays (Tzima *et al.,* 2020; Arguelles, 2022; Arguelles, 2023).

2.3. Proximate Analysis

2.3.1. *Moisture content*

Approximately 5 grams of *D. muscorum* biomass were measured into an evaporating dish. The cyanobacteria were then placed in an oven, maintained at 105 °C for a minimum of 5 hours. Subsequently, the sample was transferred to a desiccator to cool at room temperature before being weighed. The sample was subjected to heating in the oven for another 30 minutes, followed by cooling and reweighing. This process was repeated until successive weighings did not deviate by more than 0.001 gram (Arguelles et al., 2019; Arguelles, 2023).

2.3.2. *Fat content (Soxhlet method)*

Approximately 1 gram of *D. muscorum* biomass was measured into a filter paper thimble and subjected to drying in an oven for a duration of 2 hours. The treated biomass of *D. muscorum* was then transferred into an extractor, utilizing a pre-weighed Soxhlet flask, and extracted with ether for 16 hours. After extraction, the sample was extracted from the solvent, and the solvent was recovered. The Soxhlet flask containing the lipid was subsequently dried on a hot plate for 5 minutes, or until the solvent had evaporated, then allowed to cool before being weighed (Arguelles, 2023). Crude fat was calculated as follows:

$$Lipid\ Content\ (\%) = \left(\frac{Weight_{lipid}}{Weight_{sample}}\right) \times 100$$

2.3.3. *Ash content*

The biomass of *D. muscorum* underwent ignition in a muffled furnace at 550 ^{o}C for 2 hours. Subsequently, it was allowed to cool to 50°C before being transferred to a desiccator. After reaching room temperature, the sample was weighed, and then subjected to repeated ignition at 30-minute intervals until no further weight loss was observed. The calculation of ash content proceeded as follows:

$$Ash\ Content\ (\%) = \left(\frac{Weight_{ash}}{Weight_{sample}}\right) \times 100$$

2.3.4. *Crude protein (Kjeldahl) method*

Protein content was determined using Kjeldahl method, which involves analyzing the total nitrogen present and then multiplying this value by a specific factor appropriate for the sample type. The protein content was expressed as grams per 100 grams of the edible portion (Arguelles, 2023).

2.3.5. *Crude fiber content (Weende method)*

Approximately 0.3 grams of fat-free *D. muscorum* biomass were weighed out in a 500 mL Erlenmeyer flask. To this, 200 mL of H_2SO_4 under boiling conditions was added. The flask, attached to a condenser, was heated for 30 minutes, with regular rotation to ensure thorough contact of samples with the solution. After this duration, the mixture was promptly filtered through a linen cloth in a stemless funnel and washed immediately with hot distilled water until the washings were no longer acidic. The residue remaining on the cloth was rinsed back into the Erlenmeyer flask using 200 mL of boiling NaOH. The flask, again attached to a condenser, was boiled for another 30 min. The residue was then filtered once more through cloth in a funnel, washed with sterile distilled water, and quantitatively transferred back to the flask. The residue was filtered through a gooch crucible lined with a thin layer of asbestos. The contents and crucible were dried at 110°C to achieve constant weight and ignited at 600°C until carbonaceous matter has been consumed. The weight loss incurred during this process was recorded as crude fiber (Arguelles *et al.,* 2019; Arguelles, 2023).

2.3.6. *Carbohydrate content*

Carbohydrate concentration was calculated by subtracting the total of crude fat, ash, moisture, crude fiber, and crude protein from 100. Zero value was assigned to carbohydrates if the sum of fat, water, protein, and ash is more than 100 (Arguelles, 2023).

$$\%Carbohydrate = 100 - (\%Moisture\ Content + \%Protein + \%Fat + \%Ash)$$

2.4. Elemental Composition Analysis

Desmonostoc muscorum biomass was treated to dry ashing to determine the elemental composition following the standard methods (Arguelles, 2023). Initially, 1 g of *D. muscorum* biomass was dried using a muffle furnace for 5 h set at 550 °C. The drying of the algal biomass was repeated until a whitish or gray residue was obtained. The remaining residue was further dissolved in 10 mL HCl and by heating slowly the reaction mixture. The solution was shortly put to a hot plate (temperature set at 100 °C) for the remaining ash to dissolve. The collected solution was then filtered using a filter paper (Whatman) and kept in a small flask. Detection and quantification of magnesium, iron, cadmium, sodium, calcium, lead, manganese, potassium, zinc, chromium, and copper were done using an atomic absorption spectrophotometer Perkin Elmer AAnalyst 400 (Arguelles, 2022; Arguelles, 2023).

2.5. Disk Diffusion Assay

Three Gram-positive bacteria (*Listeria monocytogenes* BIOTECH 1958, Methicillin-Resistant *Staphylococcus aureus* BIOTECH 10378, and *Staphylococcus saprophyticus* BIOTECH 1802) and one Gram-negative bacteria (*Pseudomonas aeruginosa* BIOTECH 1824) were tested against *D. muscorum* crude extract using paper disc assay (Elfita *et al.,* 2019; Arguelles, 2022). Bacterial test pathogens were initially cultured in Luria Bertani broth medium and kept for 24 hours at 35 ℃ under shaking condition. Briefly, 25 mL of Mueller-Hilton agar (MHA) was poured in petri plates. A sterile swab was dipped into the inoculum tube (cell density was adjusted to equal turbidity of 0.5 McFarlands) and inoculated into the surface of dried MH agar plate. The swab was streaked three times in the entire surface of MHA agar. Three paper discs were placed on the agar surface. One paper disc was dipped in *D. muscorum* extract while the other two paper were dipped in 1000 ppm tetracycline (positive control) and methanol (negative control). Each plate was kept for 18 hours at 35°C. Inhibition zones were measured for the control antibiotic as well as *D. muscorum* extract and were expressed as percentage activity. Biocidal activity of *D. muscorum* extract was graded as strong (inhibition zone ≥ 70%), moderate (inhibition zone is 50-70%), or weak (inhibition zone < 50%).

$$\text{Antibacterial Activity (\%)} = \frac{\text{A}}{\text{B}} \text{ x } 100$$

Where: A = clear zone of *D. muscorum* extract (mm) and B = clear zone of antibiotic (mm).

2.6. Microtiter Plate Dillution Assay

Microtiter plate dillution assay was used to know the minimum bactericidal activity (MBC) and minimum inhibitory concentration (MIC) of *D. muscorum* extract against bacterial pathogens that tested positive in the disk diffusion assay (Arguelles, 2022; Arguelles, 2023). Initially, 100 µl of each culture of bacteria were mixed with *D. muscorum* extract (100 µl) at varying dilutions (7.8125 µg/mL – 1000 µg/mL) in a 96- well microtiter plate. The experimental set-up was set aside for 12 hours at 35°C. The MIC of *D. muscorum* extract is the lowest extract concentration that exhibited inhibition of bacterial growth after 12 hours incubation period. MBC of *D. muscorum* extract was assessed by placing a loopful of the test sample (MIC experimental wells that showed no visible growth of bacteria) into newly prepared TSA (tryptic soy agar). The experimental plates were kept for 24 hours at 35℃ and were examined for growth of bacteria (colony formation) for each dilution subculturing. Absence of bacterial growth would indicate that the cyanobacterial extract was bactericidal to the test organism at that particular dilution.

2.7. Statistical Analysis

The data obtained from the chemical analyses and antibacterial assays are given as means ± standard deviations of three replicates and was computed using MS Office Excel 2019.

3. RESULTS and DISCUSSION

3.1. Proximate Analysis

Cyanobacteria are important sources of macromolecules like proteins, fiber, carbohydrates, and lipids which are important for food and pharmaceutical applications (Tibbets *et al.,* 2015; Li *et al.,* 2018; Arguelles, 2021; Martinez *et al.,* 2021). The proximate composition of *D. muscorum* is shown in Table 1. Among the macromolecules, carbohydrates and proteins gained the highest concentration with an average value of 35.50 ± 0.12% and 20.19 ± 0.03%, respectively. The observed protein and carbohydrate concentration of *D. muscorum* in this study are within the reported range of *Nostoc* and *Desmonostoc* species from previous studies (Li *et al.,* 2018; Martinez *et al.,* 2021). *Desmonostoc* species are known to produce carbohydrates (heterofucans) such as glucuronic acid and galacturonic acid. The accumulation of carbohydrates in cyanobacteria is a product of the photosynthetic response of these organisms towards nutrient rich conditions wherein carbohydrate content ranges from 10-65% of the total wet weight of the algal biomass (Li *et al.,* 2018; Arguelles, 2021). The protein content of *D.*

muscorum is comparable to several microalgae reported by Tibbets *et al.,* (2015) such as *Nannochloropsis granulata* (17.9%), *Acutodesmus dimorphus* (28.9%), *Porphyridium aerugineum* (31.6%), *Neochloris oleoabundans* (30.1%), and *Phaeodactylum tricornutum* (39.6%). Generally, cyanobacterial species (such as *Desmonostoc* sp.) produce low amounts of lipid since several species of cyanobacteria possess ≤15% lipid of its total dry weight (Tibbets *et al.,* 2015 Li *et al.,* 2018; Martinez *et al.,* 2021). In this study, low lipid content was observed in *D. muscorum* which is similar to that of *N. commune* and *Tetraselmis chuii* with lipid content of 0.26 ± 0.02% and 12.3%, respectively (Tibbets *et al.,* 2015; Martinez *et al.,* 2021). Crude fiber shows the amount of indigestible component (insoluble and soluble fibers) of the algae. On the other hand, ash content reflects the amount of micronutrients present in the sample. The crude fiber and ash content of *D. muscorum* is 7.14 ± 0.13% and 16.90 ± 0.02%, respectively. The amount of crude fiber and ash observed in *D. muscorum* was comparable to those obtained from previously reported species of microalgae such as *Acutodesmus dimorphus, Chroococcus minutus, Chlorella minitussima, Spirulina* sp., and *Botryococcus braunii* (Tibbets *et al.,* 2015; Arguelles, 2021; Arguelles, 2022; Arguelles, 2023). Several factors affect the chemical composition of algal biomass such as growth condition and strain differences. Therefore, differences in proximate composition of *D. muscorum*, in contrast to other microalgal species, were observed in this study. In general, the chemical composition of *D. muscorum* are considered good sources of proteins, minerals, and carbohydrates. The high amount of these macromolecules shows the potential use of this cyanobacterial strain as functional ingredient for food and industrial application.

Table 1. Proximate analysis composition of *Desmonostoc muscorum*.

Proximate composition	Percent composition (%)
Ash Content	16.90 ± 0.02
Moisture Content	5.12 ± 0.01
Carbohydrate	35.50 ± 0.12
Crude Fiber	7.14 ± 0.13
Crude Fat	5.15 ± 0.10
Crude Protein	20.19 ± 0.03

3.2. Elemental Composition Analysis

Cyanobacteria are regarded as rich alternative source of microelements that can be harness for food and agricultural application. These microelements are important for growth and is a product of the overall metabolism of the algal cells (Arguelles, 2018). In this study, ash (minerals and other microelements) generated from complete combustion of algal biomass contains significant amounts of elemental nutrients, such as calcium, magnesium, potassium, and sodium as well as other trace metals present at different concentrations. The mineral composition and their average concentration in *D. muscorum* are shown in Table 2. The mineral distribution present in *D. muscorum* biomass was observed to be in reducing order of Ca > Mn > Mg > K > Na > Fe> Zn > Cr > Pb > Cu > Cd.

Calcium (19245.16 ±7.21ppm) is the most abundant mineral in cyanobacterial biomass, followed by manganese (6372.24 ± 2.45 ppm), magnesium (5733.52 ±117 ppm), and potassium (2723 ± 0.34 ppm). *Desmonostoc muscorum* has higher concentration of these minerals as compared to that reported to *Nostoc commune* with estimated concentration of 21151 ± 833 ppm, 125.98 ± 1.97 ppm, 1959.0 ± 36 ppm, and 1002 ± 2.0 ppm for calcium, manganese, magnesium, and potassium, respectively (Martinez *et al.,* 2021). On the other hand, *D. muscorum* showed comparable concentration of trace elements like zinc (23.78 ± 0.42 ppm), copper (1.27 ± 0.22 ppm), cadmium (0.47 ± 0.32 ppm), lead (1.71 ± 0.64 ppm), chromium (1.94 ± 0.14 ppm), and iron (576.49 ± 0.97 ppm) to that obtained for *N. commune* with estimated

concentration of 22.09±0.27 ppm, 11.88±0.69 ppm, 0.36±0.00 ppm, 3.59±0.05 ppm, 3.77±0.00 ppm, and 4202±37.0 ppm for each trace elements, respectively (Martinez *et al.,* 2021). In the Philippines, limited information is documented on the elemental analysis of microalgal biomass as compared to those reported for macroalgae (seaweeds) (Arguelles & Sapin, 2022). Cyanobacterial biomass generally contains a much lower concentration of inorganic elements (ash) as compared to seaweeds (Tibbets *et al.,* 2015; Martinez *et al.,* 2021). Variation in elemental composition concentration among diverse species of cyanobacteria is common and may be attributed to strain differences as well as culture conditions (like varying light intensity, salinity, and temperature) where the organism was grown (Arguelles, 2022).

Table 2. Concentrations and composition of micro and macro-elements of *Desmonostoc muscorum*.

Cyanobacteria	Elemental Parameter* (in ppm)				
	Ca	Mg	Na	Mn	K
Desmonostoc muscorum	19245.16 ±7.21	5733.52 ±117	1995 ±106	6372.24 ±2.45	2723 ±0.34

Cyanobacteria	Elemental Parameter* (in ppm)					
	Fe	Zn	Cu	Pb	Cr	Cd
Desmonostoc muscorum	576.49 ±0.97	23.78 ±0.42	1.27 ±0.22	1.71 ±0.64	1.94 ±0.14	0.47 ±0.32

* All experimental data are given as mean ± standard deviation (n = 3)

3.3. Antibacterial Activity

Cyanobacteria has been identified to contain promising natural products such as antibiotics and other bioactive substances (Salehghamari & Najafi, 2016; Little *et al.,* 2021). These groups of microorganisms are unexplored and thus serve as a rich pool for the isolation of active compounds (like alkaloids, phenolic compounds, pigments, fatty acids, and terpenoids) that may be of industrial and human importance. *Desmonostoc muscorum* was used to assess its biocidal activity against some medically important bacterial pathogens using the paper disc assay. The occurrence of zones of inhibition was indicative of the culture's ability to produce antibacterial substances. Results of the assay showed that *D. muscorum* possess antibacterial activities by exhibiting zones of inhibition (Table 3 and Figure 2).

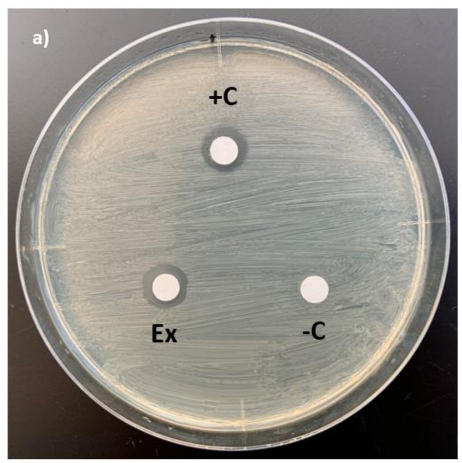
Table 3. Antibacterial activities of *Desmonostoc muscorum* extract.

Sample	Antibacterial Activity[a]			
	Gram-positive bacteria			Gram-negative bacteria
	Listeria monocytogenes BIOTECH 1958	*Staphylococcus saprophyticus* BIOTECH 1802	Methicillin-Resistant *Staphylococcus aureus* BIOTECH 10378	*Pseudomonas aeruginosa* BIOTECH 1824
Desmonostoc muscorum BIOTECH 4087	8.57 ± 0.1 (52.58**)	13.7 ± 0.7 (88.20***)	15.7 ± 0.2 (94.05***)	-
Tetracycline[b]	16.3 ± 0.3	15.53 ± 0.3	16.69 ± 0.2	19.7 ± 0.1

Note: [a]Antibacterial activity percentage (%) of algal extracts in contrast to tetracycline: inhibition zone (mm) of algal extract/ inhibition zone (mm) of antibiotic (tetracycline): *** strong inhibition (≥ 70%), **moderate inhibition (50-70%), and *weak inhibition (< 50%) against bacterial pathogen. [b]positive control (antibiotic). (-) no zone of inhibition.

The cyanobacteria exhibited inhibition zones of 13.7 ± 0.7 mm, 8.57 ± 0.1 mm, and 15.7 ± 0.2 mm against *Staphylococcus saprophyticus, Listeria monocytogenes,* and Methicillin-Resistant *Staphylococcus aureus* (MRSA)*,* respectively. The algal extract showed strong inhibition against medically important *Staphylococcus* species (*S. saprophyticus* and MRSA) and moderate inhibition against *L. monocytogenes*. On the other hand, *D. muscorum* extract showed

no antibacterial activity against *Pseudomonas aeruginosa*. The zones of inhibition of *D. muscorum* extract against Methicillin-Resistant *Staphylococcus aureus* (MRSA) is more potent than that obtained for *N. commune* (zone of inhibition = 0.9 mm), *Nostoc linkia* (zone of inhibition = 0.9 mm), and *Acutodesmus dimorphus* (zone of inhibition = 15.1 ± 0.3 mm) but is less effective to other algae such as *Oscillatoria princeps* (zone of inhibition = 18.8 mm) (Shaieb *et al.,* 2014; Yalcin *et al.,* 2022; Arguelles 2023).



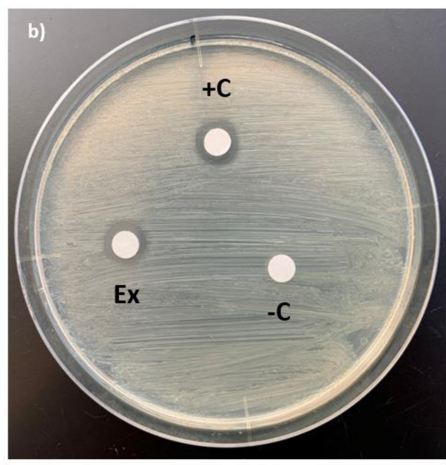


Figure 2. Inhibition zones exhibited by *Desmonostoc muscorum* methanol extract against (a) Methicillin-Resistant *Staphylococcus aureus* and (b) *Staphylococcus saprophyticus*. (-C) methanol/negative control, (+C) tetracycline/positive control, (Ex) extract.

The findings of the paper disk assay for *D. muscorum* extract were further validated by determining the MIC and MBC values of the cyanobacterial extract using microtiter plate dilution assay (Table 4). Results of the assay showed potent antibacterial activities against *S. saprophyticus* and Methicillin-Resistant *S. aureus* (MRSA) both with MIC and MBC values of 125 µg/mL and 250 µg/mL, respectively. Additionally, MIC (250 µg/mL) and MBC (500 µg/mL) values against *L. monocytogenes* showed less potent activity as compared to other tested bacterial pathogens. The antagonistic activity of *D. muscorum* extract against *L. monocytogenes* is more potent as compared to *Moorea producens* which exhibited MIC value of >500 µg/mL (Dussault *et al.,* 2016). In addition, *D. muscorum* extract showed similar antibacterial activity against *S. saprophyticus* (MIC = 125 µg/mL and MBC = 250 µg/mL) to that of *Nostoc commune* (Martinez *et al.,* 2021). However, *N. commune* did not show biocidal activity against *L. monocytegenes* whereas *D. muscorum* exhibited potent antibacterial activity. El-Sheekh, *et al.,* (2006) revealed that *D. muscorum* was able to exhibit zones of inhibition against a broad spectrum of bacteria like *S. aureus*, *Escherichia coli*, *Salmonella typhi,* and *Bacillus cereus*. These activities are said to be caused by substances such as phenolic compounds, polyketides, amides, fatty acids (palmitoleic and linoleic acids), terpenes (β-ionone, neophytadiene), alkaloids and peptides that are naturally present in the organism.

Table 4. MIC and MBC of *Desmonostoc muscorum* extract.

Bacterial Pathogens	MIC values (µg/mL)	MBC values (µg/mL)
Listeria monocytogenes BIOTECH 1958	250	500
Staphylococcus saprophyticus BIOTECH 1802	125	250
Methicillin-Resistant *Staphylococcus aureus* BIOTECH 10378	125	250
Pseudomonas aeruginosa BIOTECH 1824	>1000.00	ND

*ND = None Detected; MIC= Minimum Inhibitory Concentration; MBC= Minimum Bactericidal Concentration

Desmonostoc muscorum extract is considered more effective in inhibiting Gram-positive bacterial pathogens as compared to *Pseudomonas aeruginosa*. Gram-negative bacterial species are characterized to have thin cell wall (with peptidoglycan) and outer membrane (with specialized proteins and lipopolysaccharides). These cellular structures act as protective barrier against potent antibiotics making Gram-negative bacteria more stable than Gram-positive bacterial strain (Preisitsch *et al.,* 2015). In addition, the production of antimicrobial agents in cyanobacterial extract is also influenced by important factors during cultivation condition such as components of the growth medium, light intensity, temperature, and pH (Orhan *et al.,* 2003; Katircioglu *et al.,* 2005). Thus, it is possible that varying growth conditions of *D. muscorum* can lead to different patterns of antibacterial activities which can be a subject of future studies using this cyanobacterium. The study documented the potent antibacterial activities of *D. muscurom* against clinically important bacterial pathogens. To date, this study documents for the first time the antibacterial activities of *D. muscorum* extract against *L. monocytogenes* and Methicillin-resistant *S. aureus* (MRSA). The methanol extract of *D. muscurom* contains bioactive compounds causing these biological activities. Thus, additional studies that will target the isolation and identification of these compounds is recommended to further elucidate the biocidal activity of the extract.

4. CONCLUSION

In conclusion, the study documented that *Desmonostoc muscorum* possesses high concentration of important biomolecules such as carbohydrates, proteins, and microelements. In addition, *D. muscorum* can be utilize as potent source of antibiotics against clinically important bacterial pathogens. It is recommended that further experimental investigations should be done focusing on isolating and identifying the bioactive compounds in *D. muscorum* extract. Additionally, performing *in vivo* toxicity assays is important to substantiate the safety and efficacy of the *D. muscorum* extract for potential medical use. Further studies on the antibacterial diversity of this cyanobacterium are also recommended to assess the practicality of scaling up the production of *D. muscorum* for applications in the food and pharmaceutical industries.

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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.

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REFERENCES

Arguelles, E.D.L.R. (2023). Chemical composition and *In vitro* analysis of antibacterial Activity of *Acutodesmus dimorphus* (Turpin) P.M. Tsarenko (Scenedesmaceae, Chlorophyta). *Journal of Faculty of Pharmacy of Ankara University*, *47*(1), 169-178. https://doi.org/10.33483/jfpau.1151637

Arguelles, E.D.L.R. (2022). Total phenolic content and *In vitro* analysis of antioxidant, antibacterial, and alpha-glucosidase inhibition properties of *Chroococcus minutus* (Kützing)

Nägeli (Chroococcales, Cyanobacteria). *Journal of Faculty of Pharmacy of Ankara University*, *46*(1), 170-181. https://doi.org/10.33483/jfpau.1005986

Arguelles, E.D.L.R. (2021). Biochemical composition and bioactive properties *of Chlorella minutissima* (Chm1) as a potential source of chemical compounds for nutritional feed supplement and disease control in aquaculture. *Current Applied Science and Technology, 21*(1), 65-77.

Arguelles, E.D.L.R. (2018). Proximate analysis, antibacterial activity, total phenolic content, and antioxidant capacity of a green microalga *Scenedesmus quadricauda* (Turpin) Brébisson. *Asian Journal of Microbiology, Biotechnology, and Environmental Sciences*, *20*(1), 150–158.

Arguelles, E.D.L.R., Laurena, A.C., Monsalud, R.G., & Martinez-Goss, M.R. (2019). High lipid and protein-producing epilithic microalga, *Desmodesmus* sp. (U-AU2): a promising alternative feedstock for biodiesel and animal feed production. *Philippine Journal of Crop Science*, *44*,13–23.

Arguelles, E.D.L.R., Sapin, A.B. (2022). Proximate composition and in vitro analysis of antioxidant and antibacterial activities of *Padina boryana* Thivy. *Science, Engineering and Health Studies, 16*, 22030002. https://doi.org/10.14456/sehs.2022.2

Arguelles, E.D.L.R., Sapin, A.B. (2021). Chemical composition and bioactive properties of *Sargassum aquifolium* (Turner) C. Agardh and its potential for pharmaceutical application. *Philippine Journal Science*, *151*(S1), 9-24. https://doi.org/10.56899/151.S1.02

Cock, I.E., & Cheesman, M.J. (2023). A review of the antimicrobial properties of cyanobacterial natural products. *Molecules*, *28*(20), 7127. https://doi.org/10.3390/molecules28207127

Dussault, D., Vu, K.D., Vansach, T., Horgen, F.D., & Lacroix, M. (2016). Antimicrobial effects of marine algal extracts and cyanobacterial pure compounds against five foodborne pathogens. *Food Chemistry, 199*, 114–118. https://doi.org/10.1016/j.foodchem.2015.11.119

Elfita, Mardiyanto, Fitrya, Eka Larasati, J., Julinar, Widjajanti, H., & Muharni. (2019). Antibacterial activity of *Cordyline fructicosa* leaf extracts and its endophytic fungi extracts. *Biodiversitas, 20*(12), 3804–3812. https://doi.org/10.13057/biodiv/d201245

El-Sheekh, M.M., Osman, M.E.H, Dyab, M.A., & Amer, M.S. (2006). Production and characterization of antimicrobial active substance from the cyanobacterium *Nostoc muscorum*. *Environmental Toxicology and Pharmacology, 21*, 42-50. https://doi.org/10.1016/j.etap.2005.06.006

Ganesan, P., Reegan, A.D., David, R.H.A., Gandhi, M.R., Paulraj, M.G., Al-Dhabi, N.A., & Ignacimuthu, S. (2017). Antimicrobial activity of some actinomycetes from Western Ghats of Tamil Nadu, India. *Alexandria Journal of Medicine*, *53*(2), 101-110. https://doi.org/10.1016/j.ajme.2016.03.004

Hirata, K., Takashina, J., Nakagami, H., Ueyama, S., Murakami, K., Kanamori, T., & Miyamoto, K. (1996). Growth inhibition of various organisms by a violet pigment nostocine A, produced by *Nostoc spongiaeforme*. *Bioscience, Biotechnology, and Biochemistry, 60*, 1905-1906. https://doi.org/10.1271/bbb.60.1905

Katircioglu, H., Beyatli, Y., Aslim, B., Yüksekdag, Z., & Atici, T. (2005). Screening for antimicrobial agent production of some microalgae in freshwater. *The Internet Journal of Microbiology*, *2*(2), 1-5.

Kumar, N., Singh, R.K., Mishra, S.K., Singh, A.K., & Pachouri, U.C. (2010). Isolation and screening of soil Actinomycetes as source of antibiotics active against bacteria. *International Journal of Microbiology Research*, *2*(2), 12-16.

Li, H., Linnan, S., Chen, S., Zhao, L., Wang, H., Ding, F., Chen, H., Shi, R., Wang, Y., & Huang, Z. (2018). Physicochemical characterization and functional analysis of the polysaccharides from the edible microalga *Nostoc sphaeroides*. *Molecules*, *223*, 17-508. https://doi.org/10.3390/molecules23020508

Little, S.M., Senhorinho, G.N.A., Saleh, M., Basiliko, N., & Scott, J.A. (2021). Antibacterial compounds in green microalgae from extreme environments: a review. *Algae, 36*(1), 61-72. https://doi.org/10.4490/algae.2021.36.3.6

Martinez-Goss, M.R., Arguelles, E.D.L.R., Sapin, A.B., & Almeda, R.A. (2021). Chemical Composition and In Vitro Antioxidant and Antibacterial Properties of the Edible Cyanobacterium, *Nostoc commune* Vaucher. *Philippine Science Letters*, *14*, 25–35.

Orhan, I., Wisespongpand, P., Atıcı T., & Şener, B. (2003). Toxicity propensities of some marine and fresh-water algae as their chemical defense. *Journal of Faculty of Pharmacy of Ankara University, 32*(1), 19–29. https://doi.org/10.1501/Eczfak_0000000384

Preisitsch, M., Harmrolfs, K., Vansach, T., Pham, H.T.L., Heiden, S.E., Füssel, A., Wiesner, C., Pretsch, A., Swiatecka-Hagenbruch, M., Niedermeyer, T.H.J., Müller, R., & Mundt, S. (2015). Anti-MRSA-acting carbamidocyclophanes H–L from the Vietnamese cyanobacterium *Nostoc* sp. CAVN2. *The Journal of Antibiotics*, *68*, 165–17. https://doi.org/10.1038/ja.2014.118

Salehghamari, E., & Najafi, M. (2016). Isolation of biologically active Actinomycetes from untouched soils: a case study from Karaj district, Iran. *Progress in Biological Sciences*, *6*(1), 65-74.

Senhorinho, G.N.A., Laamanen, C.A., & Scott, J.A. (2018). Bioprospecting freshwater microalgae for antibacterial activity from water bodies associated with abandoned mine sites. *Phycologia, 57*, 432–439. https://doi.org/10.2216/17-114.1

Shaieb, F.A., Issa, A.A., & Meragaa, A. (2014). Antimicrobial activity of crude extracts of cyanobacteria *Nostoc commune* and *Spirulina platensis*. *Archives of Biomedical Sciences*, *2*(2), 34-41.

Tibbetts, S.M., Milley, J.E., & Lall, S.P. (2015). Chemical composition and nutritional properties of freshwater and marine microalgal biomass cultured in photobioreactors. *Journal of Applied Phycology, 27*, 1109–1119. https://doi.org/10.1007/s10811-014-0428-x

Tzima, K., Brunton, N.P., Rai, D.K. (2020). Evaluation of the impact of chlorophyll removal techniques on polyphenols in rosemary and thyme by-products. *Journal of Food Biochemistry*, *44*(3), e13148. https://doi.org/10.1111/jfbc.13148

Yalçın, D., Erkaya, İ.A., & Erdem, B. (2022). Antimicrobial, antibiofilm potential, and anti-quorum sensing activity of silver nanoparticles synthesized from cyanobacteria *Oscillatoria princeps*. *Environmental Science and Pollution Research, 29*, 89738-89752. https://doi.org/10.1007/s11356-022-22068-y

Yasin, D., Zafaryab, Md., Ansari, S., Ahmad, N., Khan, N.F., Zaki, A., Rizvi, M.M.A. & Fatma, T. (2019). Evaluation of antioxidant and anti-proliferative efficacy of *Nostoc muscorum* NCCU-442. *Biocatalysis and Agricultural Biotechnology*, *17*, 284-293. https://doi.org/10.1016/j.bcab.2018.12.001

Żymańczyk-Duda, E., Samson, S.O., Brzezińska-Rodak, M., & Klimek-Ochab, M. (2022). Versatile applications of cyanobacteria in biotechnology. *Microorganisms, 10*(12), 2318. https://doi.org/10.3390/microorganisms10122318

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Effects of nitric oxide on composition of the isolated essential oil from *Satureja hortensis* L. (Lamiaceae), under the cadmium stress

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Abstract: Cadmium (Cd) is one of the heavy metals that cause environmental pollution and biochemical changes in plants grown in contaminated soils. In plants, sodium nitroprusside is used as a nitric oxide (NO) release agent. In this research, a glasshouse pot experiment was conducted to examine the effect of exogenous NO on the essential oil composition of the savory plant, *Satureja hortensis*, under the Cd stress. For this, the plants were treated by different levels of Cd concentration including 0 (control), 75, 100, and 150 µM in the contaminated soil. Plants were also foliar sprayed with concentrations of 0 (control), 50, 100, and 200 µM NO. The results indicated that carvacrol was the main compound in all examined essential oils. Also, there were significant differences among the essential compounds under treatments of Cd and NO. Moreover, the differences among minor constituents were not significant in most of treatments. In apposite, carvacrol (approximately 60% of total volume) showed a significant difference than the others. The results indicated the role of exogenous agents on the changes of essential oil constituents in *S. hortensis*.

1. INTRODUCTION

One of the major environmental concerns is the contamination of soils by heavy metals, which have severe negative effects on production and safety of plants. Cd is one of the common pollutants which have toxic effects on all living organisms (Benavides *et al*., 2005). Cd interferes with functionally active ions on enzyme sites when inhibits many key enzymes involved in various metabolic pathways including secondary metabolism (Andresen & Küpper, 2013). In the presence of polyphenolic compounds, *Satureja hortensis* L. essential oil has antioxidant, antimicrobial, antiparasitic, pesticidal, anti-inflammatory, antinociceptive, hepatoprotective, and anticancer effects (Fierascu *et al*., 2018). Plant secondary metabolism can seriously affect the quality and efficacy of valuable natural products such as essential oils derived from medicinal plants (Azizollahi *et al*., 2019). Cd toxicity induces production of reactive oxygen species (ROS) at the cellular level and impairs redox homeostasis (Asopa *et al*., 2017). During normal plant growth, ROS molecules are produced and detoxified in balance due to metabolic processes. Abiotic stresses such as heavy metal toxicity disturb the balance

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between ROS production and removal which causes membrane peroxidation, enzyme inhibition, as well as DNA, RNA, and protein damage (Anjitha *et al*., 2021). In response to stressful metal concentrations, plants employ various defense strategies to detoxify ROS (Haider *et al*., 2021). Plants can accumulate secondary metabolites when metal-induced biosynthesis pathways are activated. Plants synthesize them when they undergo physiological changes that require primary metabolism (Bali *et al*., 2020). Although plants are sessile organisms and have no immune system, they have adopted various defense strategies including a wide range of secondary metabolites to overcome environmental stresses (Anjitha *et al*., 2021). In plants, the secondary metabolites fall into three categories based on their biosynthesis pathways. These include terpenes, phenolic compounds, and nitrogen-containing compounds (Ashraf *et al*., 2018).

NO (Nitric oxide) is a gaseous molecule. It had variable effects on different organisms' strata and animals' neurotransmitters. NO also has significant importance in plant signaling which regulates the growth of plants and development processes such as germination, formation of roots, movement of stomata, maturation, and plant defense (Azizi *et al*., 2021; Kumar & Ohri, 2023). Essential oils are natural products with economic potential that are formed mostly of terpenes (Asadi *et al*., 2018, 2021). These compounds play a key role in human activities including medical treatment and industrial manufacturing. There are extensive studies on essential oils as natural products; but the effects of environment on their production and composition are poorly understood (Ribeiro *et al*., 2019).

The summer savory, *Satureja hortensis*, (Figure 1) is one of more than 30 species of plants in the family Lamiaceae that grow in the Eastern Mediterranean (Şahin *et al*., 2003). It is an old plant that has been used for vegetable, medicinal, and aromatic purposes (Mohtashami *et al*., 2018). Essential oil of summer savory is used in the food (conservation and beverage) and pharmaceutical industries (Mihajilov-Krstev *et al*., 2009). It is applied in Türkiye for curing high blood pressure and as an antispasmodic agent against upper respiratory tract problems and inflammations of reproductive system (Selvi *et al*., 2022). Its essential oil contains higher carvacrol and thymol contents that display strong antimicrobial impacts (Mohtashami *et al*., 2021; Azizi & Asadi, 2024). Carvacrol has many biological effects: it is antiseptic, anti-inflammatory, deworming, antioxidant, analgesic, antifungal, and antibacterial as well as yeast inhibitor (Fierascu *et al*., 2018).

Figure 1. Above-ground parts of *S. hortensis.*

The other compounds also have similar importance. Changes in the secondary metabolites of medicinal plants treated with chemical compounds are very important. Accordingly, the present research was designed. We have investigated the constituents of *S. hortensis* under Cd and NO treatments, which are important from different aspects.

2. MATERIAL and METHODS

2.1. Plant Material and Experimental Designs

A factorial plot experiment was conducted in the form of a completely randomized design (CRD) with three replications in the greenhouse of the Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran. The main aim was to investigate the response of NO foliar spraying on the content of essential oil in *S. hortensis* under Cd stress. $CdCl_2$ and sodium nitroprusside (NO) were obtained from Merck (Darmstadt, Germany). Soil pollution with different concentrations of $CdCl_2$ solution (0, 75, 100, and 150 mM) is considered as Cd stress under field capacity moisture. The soils were exposed to wet/dry cycles for 4 months to prove close to natural conditions and long-term contamination.

Savory seeds were purchased from Tehran Agricultural Research Station and sterilized with sodium hypochlorite; then, rinsed with the deionized water. The seeds were planted in the contaminated soil. After emergence of the seedling stage, foliar spraying of NO solution was done in four concentrations (0, 50, 100, and 200 µM), under three replications. Spraying was performed in three stages (final amount of 200 ccs for each pot) and every two weeks. The first spraying was done after appearance of two true leaves in the seedling stage (Azizi *et al*., 2021).

2.2. Essential Oil Isolation

The aerial parts of the savory plant, which contained the most essential oil, were separated from each sample and dried at room temperature (about 25°C) under shade. Then, were powdered by an electric mill and 50 grams of each sample were added to 500 ml of the distilled water. Their essential oils were isolated by a Clevenger apparatus at the temperature of 100 °C. The water of essential oils was deleted by Na_2So_4 and the pure essential oils stored at 4 °C in sealed brown vials until the chemical analysis (Asadi *et al*., 2018, 2019).

2.3. Chemical Analysis of The Isolated Essential Oils

To determine the quantitative and qualitative components of the essential oils, we used the Agilent Technologies 7890B (manufactured in the USA) gas chromatography with an HP-5MS column, length 30 m, diameter 0.25 mm, and film thickness 0.25 mm. Temperature program held at 350 °C. The ranges were determined by using mass data, Kovats index, and retention time. Each essential oil constituent was detected based on patterns of range refraction compared to two standard libraries (Adams, 2001).

2.4. Statistical Analysis

In the present research, three replicates were considered for all treatments. Then, the data were examined for normality status. Finally, the normal data were analyzed by one-way ANOVA and their means were compared by Tukeys' test at probability level $p<0.05$ by using SPSS (version 22) software.

3. RESULTS

3.1. Cd Treatments with 0 µM of NO

The effects of Cd concentrations (0, 75, 100, and 150 µM) with 0 µM of NO on *S. hortensis* essential oil are shown in Table 1. In all of them, carvacrol was the main constituent. Under 0 µM×0 µM (Cd×NO) treatment, the differences among most of the compounds were not significant ($F_{17, 36}$= 8.93). About 75 µM×0 µM (Cd×NO) treatment, carene and limonene were not available in the essential oil structure ($F_{17, 36}$= 14.55). About 100 µM×0 µM (Cd×NO) treatment, carene showed the lowest percentage, and limonene with thymyl acetate were not available ($F_{17, 36}$= 17.06). Finally, under 150 µM×0 µM (Cd×NO) treatment, majority of differences were significant when carene had the lowest percentage in the essential oil ($F_{17, 36}$= 27.93).

Table 1. Effects of different Cd concentrations with 0 µM of NO on *S. hortensis* essential oil.

Compound	Cadmium × Nitric Oxide			
	0 µM × 0 µM	75 µM × 0 µM	100 µM × 0 µM	150 µM × 0 µM
α-Pinene	1.02 ± 0.11 [e]	1.06 ± 0.12 [efg]	1.36 ± 0.05 [ef]	1.27 ± 0.04 [de]
α-Terpinene	4.51 ± 0.10 [d]	4.45 ± 0.11 [d]	5.19 ± 0.15 [d]	5.57 ± 0.07 [d]
α-Thujene	1.40 ± 0.15 [e]	1.26 ± 0.08 [e]	1.67 ± 0.23 [e]	1.46 ± 0.03 [e]
ß-Bisabolene	0.11 ± 0.11 [e]	0.23 ± 0.11 [gh]	0.31 ± 0.19 [f]	0.30 ± 0.05 [g]
ß-Myrcene	1.42 ± 0.06 [e]	1.20 ± 0.06 [ef]	1.72 ± 0.14 [e]	1.31 ± 0.14 [de]
ß-Pinene	0.40 ± 0.03 [e]	0.41 ± 0.03 [fgh]	0.59 ± 0.02 [ef]	0.43 ± 0.07 [efg]
γ-Terpinene	20.22 ± 0.75 [b]	19.20 ± 0.23 [b]	22.28 ± 0.37 [b]	23.42 ± 0.29 [b]
Benzene	7.53 ± 0.46 [c]	6.07 ± 0.58 [c]	7.38 ± 0.83 [c]	8.37 ± 0.32 [c]
Camphene	0.22 ± 0.07 [e]	0.04 ± 0.02 [h]	0.07 ± 0.03 [f]	0.07 ± 0.02 [g]
Carvacrol	59.34 ± 0.57 [a]	63.92 ± 0.36 [a]	57.02 ± 0.49 [a]	55.04 ± 0.65 [a]
Carene	0.06 ± 0.06 [e]	0.00 ± 0.00 [h]	0.03 ± 0.03 [f]	0.03 ± 0.03 [g]
Caryophyllene	0.39 ± 0.03 [e]	0.23 ± 0.11 [gh]	0.30 ± 0.07 [f]	0.14 ± 0.07 [g]
Cyclohexen	0.89 ± 0.39 [e]	0.14 ± 0.03 [h]	0.04 ± 0.04 [f]	0.22 ± 0.11 [g]
Limonene	0.00 ± 0.00 [e]	0.00 ± 0.00 [h]	0.00 ± 0.00 [f]	0.56 ± 0.40 [efg]
Octatriene	0.03 ± 0.03 [e]	0.06 ± 0.03 [h]	0.08 ± 0.04 [f]	0.09 ± 0.04 [g]
Phellandrene	0.52 ± 0.12 [e]	0.36 ± 0.02 [fgh]	0.39 ± 0.02 [f]	0.43 ± 0.02 [efg]
Sabinene	0.09 ± 0.05 [e]	0.11 ± 0.05 [h]	0.08 ± 0.04 [f]	0.17 ± 0.08 [g]
Thymyl acetate	0.42 ± 0.21 [e]	0.03 ± 0.03 [h]	0.00 ± 0.00 [f]	0.13 ± 0.08 [g]

The values in each column with different letters show significant differences (Tukey's test, $p<0.05$).

3.2. Cd Treatments with 50 µM of NO

Cd treatments (0, 75, 100, and 150 µM) with 50 µM of NO on the essential oil of *S. hortensis* were investigated (Table 2). Among all of them, carvacrol was the main constituent. Under 0 µM×50 µM (Cd×NO), carvacrol and limonene had the highest and lowest percentages on total ($F_{17, 36}$= 22.58). Under 75 µM×50 µM (Cd×NO) treatment, carvacrol had the highest percentage while camphene and carene showed the lowest percentages ($F_{17, 36}$= 23.58). Moreover, about 100 µM×50 µM (Cd×NO) treatment, most of the differences among compounds were significant ($F_{17, 36}$= 4.12). Finally, by treatment of 150 µM×50 µM (Cd×NO), carvacrol and γ-terpinene showed higher percentages compared to the others, respectively ($F_{17, 36}$= 18.79).

Table 2. Effects of different Cd concentrations with 50 µM of NO on *S. hortensis* essential oil.

Compound	Cadmium × Nitric Oxide			
	0 µM × 50 µM	75 µM × 50 µM	100 µM × 50 µM	150 µM × 50 µM
α-Pinene	0.85 ± 0.16 [df]	1.30 ± 0.03 [d]	1.20 ± 0.14 [def]	1.23 ± 0.04 [e]
α-Terpinene	4.42 ± 0.10 [c]	4.67 ± 0.09 [c]	5.23 ± 0.23 [d]	5.59 ± 0.32 [c]
α-Thujene	1.29 ± 0.11 [d]	1.60 ± 0.10 [d]	1.88 ± 0.19 [e]	1.66 ± 0.04 [de]
ß-Bisabolene	0.24 ± 0.08 [e]	0.20 ± 0.08 [d]	0.19 ± 0.11 [g]	0.30 ± 0.08 [fg]
ß-Myrcene	1.36 ± 0.01 [d]	1.27 ± 0.06 [d]	1.79 ± 0.34 [de]	1.75 ± 0.02 [d]
ß-Pinene	0.43 ± 0.02 [df]	0.40 ± 0.05 [d]	0.64 ± 0.06 [fg]	0.58 ± 0.05 [f]
γ-Terpinene	0.43 ± 0.03 [b]	20.51 ± 0.65 [b]	25.17 ± 0.22 [b]	23.21 ± 0.18 [b]
Benzene	4.89 ± 0.15 [c]	4.58 ± 0.78 [c]	6.50 ± 0.75 [c]	5.64 ± 0.15 [c]
Camphene	0.10 ± 0.01 [e]	0.03 ± 0.01[d]	0.07 ± 0.05 [g]	0.12 ± 0.02 [fg]
Carvacrol	62.52 ± 0.35 [a]	63.64 ± 0.83 [a]	56.73 ± 0.41 [a]	57.02 ± 0.14 [a]
Carene	0.02 ± 0.02 [e]	0.03 ± 0.03 [d]	0.03 ± 0.03 [g]	0.05 ± 0.05 [fg]
Caryophyllene	0.12 ± 0.13 [e]	0.37 ± 0.03 [d]	0.42 ± 0.08 [g]	0.25 ± 0.06 [fg]
Cyclohexen	1.26 ± 0.14 [d]	0.19 ± 0.01 [d]	0.63 ± 0.24 [fg]	0.21 ± 0.07 [fg]
Limonene	0.00 ± 0.00 [e]	0.07 ± 0.07 [d]	0.37 ± 0.22 [g]	0.18 ± 0.12 [fg]
Octatriene	0.03 ± 0.03 [e]	0.13 ± 0.06 [d]	0.14 ± 0.08 [g]	0.00 ± 0.00 g
Phellandrene	0.43 ± 0.06 [e]	0.36 ± 0.04 [d]	0.44 ± 0.06 [g]	0.43 ± 0.04 [fg]
Sabinene	0.09 ± 0.06 [e]	0.20 ± 0.10 [d]	0.40 ± 0.27 [g]	0.16 ± 0.02 [fg]
Thymyl acetate	0.11 ± 0.11 [df]	0.07 ± 0.07 [d]	0.05 ± 0.05 [g]	0.03 ± 0.03 [fg]

The values in each column with different letters show significant differences (Tukey's test, $p<0.05$).

3.3. Cd Treatments with 100 µM of NO

The effects of Cd concentrations (0, 75, 100, and 150 µM) with 100 µM of NO on *S. hortensis* essential oil are shown in Table 3. Among all of the investigated essential oils, carvacrol was a major constituent. About the treatment of 0 µM×100 µM (Cd×NO), carvacrol and octatriene showed the highest and lowest percentages compared to the others ($F_{17, 36}$= 5.47). Moreover, under 75 µM×100 µM (Cd×NO) treatment, most of differences among constituents were not significant ($F_{17, 36}$= 5.95). On 100 µM×100 µM (Cd×NO) treatment, carvacrol and γ-terpinene showed higher percentages than the others ($F_{17, 36}$= 5.12). Finally, by treatment of 150 µM×100 µM (Cd×NO), carvacrol had the highest while octatriene showed the lowest percentages compared to the other compounds ($F_{17, 36}$= 10.22).

Table 3. Effects of different Cd concentrations with 100 µM of NO on *S. hortensis* essential oil.

Compound	Cadmium × Nitric Oxide			
	0 µM × 100 µM	75 µM × 100 µM	100 µM × 100 µM	150 µM × 100 µM
α-Pinene	0.79 ± 0.06 ^{e}	1.32 ± 0.12 ^{e}	1.27 ± 0.13 ^{ef}	0.90 ± 0.11 ^{fg}
α-Terpinene	4.60 ± 0.11 ^{c}	4.43 ± 0.06 ^{c}	5.84 ± 0.23 ^{c}	6.53 ± 0.14 ^{c}
α-Thujene	1.43 ± 0.14 ^{e}	1.35 ± 0.09 ^{e}	1.37 ± 0.08 ^{ef}	1.74 ± 0.09 ^{e}
ß-Bisabolene	0.20 ± 0.10 ^{e}	0.38 ± 0.07 ^{e}	0.56 ± 0.24 ^{e}	0.35 ± 0.05 ^{fg}
ß-Myrcene	1.31 ± 0.14 ^{e}	1.34 ± 0.06 ^{e}	2.29 ± 0.22 ^{e}	1.85 ± 0.19 ^{e}
ß-Pinene	0.52 ± 0.03 ^{e}	0.47 ± 0.05 ^{e}	0.46 ± 0.03 ^{e}	0.72 ± 0.02 ^{fg}
γ-Terpinene	21.42 ± 0.51 ^{b}	21.50 ± 0.73 ^{b}	22.49 ± 0.44 ^{b}	24.08 ± 0.12 ^{b}
Benzene	3.30 ± 0.32 ^{d}	3.27 ± 0.31 ^{d}	4.12 ± 0.45 ^{d}	2.85 ± 0.29 ^{d}
Camphene	0.05 ± 0.03 ^{e}	0.10 ± 0.01 ^{e}	0.12 ± 0.00 ^{e}	0.18 ± 0.06 ^{fg}
Carvacrol	63.94 ± 0.77 ^{a}	62.69 ± 0.83 ^{a}	58.58 ± 0.93 ^{a}	57.28 ± 0.28 a
Carene	0.27 ± 0.04 ^{e}	0.07 ± 0.04 ^{e}	0.10 ± 0.05 ^{e}	0.18 ± 0.12 ^{fg}
Caryophyllene	0.15 ± 0.07 ^{e}	0.31 ± 0.08 ^{e}	0.37 ± 0.00 ^{e}	0.31 ± 0.02 ^{fg}
Cyclohexen	0.26 ± 0.13 ^{e}	0.15 ± 0.05 ^{e}	0.05 ± 0.05 ^{e}	0.15 ± 0.00 ^{fg}
Limonene	0.34 ± 0.20 ^{e}	0.83 ± 0.16 ^{e}	0.66 ± 0.35 ^{e}	0.93 ± 0.46 ^{f}
Octatriene	0.07 ± 0.04 ^{e}	0.16 ± 0.04 ^{e}	0.10 ± 0.05 ^{e}	0.04 ± 0.04 ^{g}
Phellandrene	0.46 ± 0.05 ^{e}	0.50 ± 0.09 ^{e}	0.43 ± 0.03 ^{e}	0.45 ± 0.06 ^{fg}
Sabinene	0.21 ± 0.07 ^{e}	0.29 ± 0.08 ^{e}	0.31 ± 0.13 ^{e}	0.26 ± 0.04 ^{fg}
Thymyl acetate	0.20 ± 0.12 ^{e}	0.00 ± 0.00 ^{e}	0.15 ± 0.10 ^{e}	0.24 ± 0.12 ^{fg}

The values in each column with different letters show significant differences (Tukey's test, p<0.05).

3.4. Cd Treatments with 200 µM of NO

Cd treatments (0, 75, 100, and 150 µM) with 200 µM of NO on the essential oil of *S. hortensis* were investigated (Table 4). Among all of them, carvacrol was the main constituent. On 0 µM×200 µM (Cd×NO) treatment, carvacrol and carene showed the highest and lowest percentages ($F_{17, 36}$= 16.74). Under treatment of 75 µM×200 µM (Cd×NO), both compounds (carvacrol and carene) showed similar positions ($F_{17, 36}$= 6.06). Furthermore, on the treatment of 100 µM×200 µM (Cd×NO), differences among most of compounds were not significant ($F_{17, 36}$= 16.44). Finally, in the treatment of 150 µM×200 µM (Cd×NO), carvacrol had the highest while limonene showed the lowest percentage than the others ($F_{17, 36}$= 10.40).

Table 4. Effects of different Cd concentrations with 200 µM of NO on *S. hortensis* essential oil.

Compound	Cadmium × Nitric Oxide			
	0 µM × 200 µM	75 µM × 200 µM	100 µM × 200 µM	150 µM × 200 µM
α-Pinene	0.86 ± 0.05 [ef]	1.44 ± 0.72 [d]	1.46 ± 0.06 [ef]	1.25 ± 0.11 [ef]
α-Terpinene	4.49± 0.10 [c]	5.23 ± 0.27 [c]	5.48 ± 0.29 [c]	5.99 ± 0.20 [c]
α-Thujene	1.53 ± 0.27 [fg]	1.57 ± 0.07 [d]	2.11 ± 0.31 [de]	1.72 ± 0.07 [de]
ß-Bisabolene	0.35 ± 0.04 [fg]	0.47 ± 0.08 [d]	0.47 ± 0.12 [fg]	0.35 ± 0.12 [g]
ß-Myrcene	1.29 ± 0.08 [de]	1.52 ± 0.22 [d]	2.68 ± 0.32 [g]	1.96 ± 0.06 [e]
ß-Pinene	0.46 ± 0.10 [fg]	0.45 ± 0.08 [d]	0.71 ± 0.03 [fg]	0.78 ± 0.08 [fg]
γ-Terpinene	19.32 ± 0.15 [b]	22.03 ± 0.53 [b]	22.97 ± 0.51 [b]	24.41 ± 0.44 [b]
Benzene	2.92 ± 0.36 [d]	4.60 ± 0.89 [c]	4.00 ± 0.15 [d]	4.26 ± 0.24 [d]
Camphene	0.11 ± 0.02 [g]	0.10 ± 0.02 [d]	0.16 ± 0.01 [g]	0.16 ± 0.05 [g]
Carvacrol	65.96 ± 0.08 [a]	59.32 ± 0.42 [a]	55.31 ± 0.36 [a]	57.20 ± 0.26 [a]
Carene	0.09 ± 0.09 [g]	0.06 ± 0.06 [d]	0.22 ± 0.06 [g]	0.08 ± 0.08 [g]
Caryophyllene	0.32 ± 0.02 [fg]	0.32 ± 0.05 [d]	0.39 ± 0.02 [fg]	0.34 ± 0.04 [g]
Cyclohexen	0.17 ± 0.04 [fg]	0.17 ± 0.02 [d]	0.21 ± 0.12 [g]	0.19 ± 0.10 [g]
Limonene	0.71 ± 0.20 [fgh]	0.26 ± 0.26 [d]	1.12 ± 0.33 [fg]	0.00 ± 0.00 [g]
Octatriene	0.10 ± 0.01 [g]	0.17 ± 0.04 [d]	0.19 ± 0.04 [g]	0.07 ± 0.03 [g]
Phellandrene	0.44 ± 0.07 [fg]	0.60 ± 0.10 [d]	0.59 ± 0.05 [fg]	0.50 ± 0.01 [g]
Sabinene	0.21 ± 0.07 [fg]	0.40 ± 0.15 [d]	0.44 ± 0.16 [fg]	0.09 ± 0.04 [g]
Thymyl acetate	0.19 ± 0.10 [fg]	0.14 ± 0.07 [d]	0.10 ± 0.10 [g]	0.19 ± 0.09 [g]

The values in each column with different letters show significant differences (Tukey's test, $p<0.05$).

4. DISCUSSION

Regarding the effects of NO and heavy metals on secondary metabolites, limited studies have been conducted. NO in plants is absorbed endogenously, by the surrounding atmosphere and soil. The quality of this gaseous composition differs depending on fluctuations in plant and environment. Proper plant growth, vegetative development, and reproduction require hormonal activity with an antioxidant network, as well as maintaining the concentration of active oxygen and nitrogen species on certain ranges. Plants often face abiotic stress conditions such as nutrient deficiency, salinity, drought, high UV radiation, extreme temperatures, and heavy metal stress which can affect growth processes and lead to their growth limitation. The ability of plants to respond and survive under environmental stress involves sensing and signaling events which NO becomes a critical component when mediates hormonal actions, interacts with reactive oxygen species, modulates gene expression, and activity of proteins (Simontacchi *et al*., 2015).

Tripathi *et al*., (2017) evaluated the effect of NO (100 µM) on the stress of nano ZnO in wheat plants. In their study, NO declined accumulation of zinc in vascular tissues and led to a decrease in oxidative stress in wheat plants. Also, the supply of NO led to regulation of antioxidants (ascorbate-glutathione) and non-antioxidant enzymes (ascorbate and glutathione). In this way, the stress of nano ZnO in wheat plants was reduced. Akladious and Mohamed (2017) studied the effects of NO (20 µM) on the mitigation of Zn toxicity in sunflower plants. Their study showed that treatment of NO increased the content of ascorbic acid glutathione and antioxidant enzymes. NO supply increased sunflower oil quality due to the enhancement of unsaturated fatty acid contents. Prieto *et al*., (2007) studied the essential oil of *Origanum vulgare* L. and *Satureja montana* L. under peroxynitrite and concluded that there was a significant difference with decreasing 3-nitrotyrosine. Also, production of compounds was inhibited by peroxynitrite induced with malondialdehyde. Furthermore, thymol and carvacrol inhibited the formation of nitrotyrosine and reduced malondialdehyde. Additionally, p-cymene and γ-terpinene were inactive in both assessments. Their results indicated that thymol and carvacrol had main roles in the prevention of toxic product formation in *O. vulgare*. The type of treatment applied and plant species in the present study were different from our study. These

parameters are among the main factors affecting plant secondary metabolites, which should be given special attention.

The effects of salt stress on the biochemical parameters of *S. hortensis* were evaluated by Najafi and Khavari-Nejad (2010). They found that the main constituents were carvacrol and γ-terpinene in the control plants. Under NaCl treatment, with concentration increasing, carvacrol increased when γ-terpinene reduced. In all treated plants by NaCl, growth parameters were negatively reduced. Finally, with salinity increasing, carvacrol increased which is useful in medicinal applications. Despite differences in the type of treatment applied, the dominant composition in the species (carvacrol) was the same, this constituent has a lot of differences with the others, and its dominance did not lost with the applied changes. Vafa *et al*., (2015) studied the effects of Nano Zinc and Humic acid on *S. hortensis* and found that the highest essential oil content was gained under N_4. Also, its minimum content was observed under treatment by N_4H_4. Said-Al Ah *et al*., (2016) evaluated the effect of N and P on the composition of essential oil from *S. montana* when found that carvacrol was major. The other important compounds were p-cymene, γ-terpinene, linalool, thymol, and β-Caryophyllene. The highest percentage of carvacrol was recorded from plants harvested at 2nd cut and fertilized by 50 kgN+30 kg P/fed. They concluded that essential oil constituents are affected by N and F under the first and second cuts. In the study, we did not examine the effects of fertilizer treatments on the essential oil of this medicinal plant; but, this case can be considered as a new field of study about the secondary metabolites in this plant. The effect of methanolic extracts integration from *Tanacetum parthenium* (L.) and *S. Montana* on antioxidant capacity and NO was evaluated by Bahramnezhad *et al*., (2021). They concluded that the combination led to a significant increase in total antioxidant activity compared to the control. Also, in the grease test, their combination significantly reduced NO production compared to the control group. The results of their studies were in agreement with our results, which showed a clear effect of NO treatment on the changes of various compounds in plants.

Regarding heavy metals, Mumivand *et al*., (2011) studied the essential oil content from *S. hortensis* under calcium carbonate and N applications and found that interaction of N and $CaCO_3$ was significant in the essential oil contents. GC-MS results showed that compositions of *S. hortensis* such as carvacrol, γ-terpinene, and β-bisabolene did not change with the compound application. Karimi *et al*., (2013) evaluated Cd accumulation in *S. hortensis* when reported that arsenic, cadmium, and mercury were observed in roots rather than shoots in artichoke and savory. Artichoke had higher uptake from metalloids and heavy metals, a factor of bioaccumulation, and translocation efficiencies from root to shoot than savory. Accordingly, artichokes had a greater accumulating capacity. Moreover, phytoextraction of metals by artichoke can be applied to clean the soils from contamination by heavy metals. Different heavy metals have variable effects on plant secondary compounds and this has been proven in different studies. Naturally, this case should be considered by researchers in the soils contaminated with these metals. In another study, the effect of organic fertilizers on the essential oil composition of *S. hortensis* was studied by Esmaielpour *et al*., (2018). They reported that the highest and lowest contents from its essential oil were in plants under vermicompost 30% and unwashed spent mushroom compost, respectively. Also, its main constituents were carvacrol and γ-terpinene. The highest level of those was observed in plants grown on substrates containing 40% and 20% washed spent mushrooms, respectively. In our studies, the role of organic fertilizers was not investigated; however, this is a new aspect that should be considered. Although, despite the application of different treatments, the main composition of the essential oil from this medicinal plant did not change, this confirms our results.

Azizollahi *et al*., (2019) evaluated Cd accumulation in *S. hortensis* and reported that its main constituent was carvacrol, which showed quality under treatment by this heavy metal. *S. hortensis* can also be considered an invaluable alternative crop for mildly Cd-contaminated soils. Besides, because of the high potential from Cd accumulation in the roots, *S. hortensis* can

be a suitable tool for phytostabilization purposes. Finally, Memari-Tabrizi *et al*., (2021) investigated foliar application of silicon nanoparticles to mitigate Cd stress on essential oil constituents from *S. hortensis* and reported the main of them were carvacrol, γ-terpinene, p-cymene, and thymol with changes in their concentrations under Cd and Si-NPs. Azizi & Asadi (2024) studied the effect of selenium on essential oil of *S. hortensis* under the cadmium stress and concluded that carvacrol being major constituent in most of analyses. Furthermore differences among minor constituents in most of the treatments were not significant which is in direction with this study.

5. CONCLUSION

This study indicated that NO treatment has obvious effects on *S. hortensis* essential oil compounds under Cd stress conditions, although the major constituent was not changed due to its high difference with the others. In this way, carvacrol (approximately 60% of total volume) showed a significant difference than the others. These studies recommend improving the biological position of *S. hortensis* under Cd stress. For this, the authors of this article encourage other researchers to examine the essential oils under the treatment of various agents. They hope that it will be possible to determine the conditions in which the highest amount of these compounds with the highest percentage can be isolated.

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

Iraj Azizi: Investigation, Methodology, Supervision, and Validation. **Mohammad Asadi:** Resources, Visualization, Software, Formal Analysis, and Writing Original draft.

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REFERENCES

Adams, R.P. (2001). Identification of essential oil components by gas chromatography-quadrupole mass spectroscopy. Allured publishing corporation.

Akladious, S.A., & Mohamed, H.I. (2017). Physiological role of exogenous nitric oxide in improving performance, yield and some biochemical aspects of sunflower plant under zinc stress. *Acta Biologica Hungarica*, *68*(1), 101-114.

Andresen, E., & Küpper, H. (2013). Cadmium toxicity in plants. Cadmium: from toxicity to essentiality. *Metal Ions Life Science, 11*, 395-413.

Anjitha, K.S., Sameena, P.P., & Puthur, J.T. (2021). Functional aspects of plant secondary metabolites in metal stress tolerance and their importance in pharmacology. *Plant Stress, 2*, 100-138. https://doi.org/10.1016/j.stress.2021.100038

Asadi, M., Nouri-Ganbalani, G., Rafiee-Dastjerdi, H., Hassanpour, M., & Naseri, B. (2018). The effects of *Rosmarinus officinalis* L. and *Salvia officinalis* L. (Lamiaceae) essential oils on demographic parameters of *Habrobracon hebetor* Say (Hym.: Braconidae) on *Ephestia kuehniella* Zeller (Lep.: Pyralidae) Larvae. *Journal of Essential Oil Bearing Plants, 21*(3), 169-182. https://doi.org/10.1080/0972060X.2018.1491331

Asadi, M., Rafiee-Dastjerdi, H., Nouri-Ganbalani, G., Naseri, B., & Hassanpour, M. (2019). Insecticidal activity of isolated essential oils from three medicinal plants on the biological control agent, *Habrobracon hebetor* Say (Hymenoptera: Braconidae). *Acta Biologica Szegediensis, 63*(1), 63-68. https://doi.org/10.14232/abs.2019.1.63-68

Asadi, M., Nouri-Ganbalani, G., Rafiee-Dastjerdi, H., Vahedi, H., Hassanpour, M., & Naseri, B. (2021). Effects of plant essential oils on the changes of digestive enzymes in the

ectoparasitoid, *Habrobracon hebetor* Say, with description of its digestive tube. *Arthropod-Plant Interactions, 15*(6), 929-935.

Ashraf, M.A., Iqbal, M., Rasheed, R., Hussain, I., Riaz, M., & Arif, M.S. (2018). Environmental stress and secondary metabolites in plants: an overview. *Plant Metabolites and Regulation Under Environmental Stress,* 153-167, https://doi.org/10.1016/B978-0-12-812689-9.00008-X

Asopa, P.P., Bhatt, R., Sihag, S., Kothari, S.L., & Kachhwaha S. (2017). Effect of cadmium on physiological parameters of cereal and millet plants-a comparative study. *International Journal of Phytoremediation, 19*(3), 225-230.

Azizi, I., Esmaielpour, B., & Fatemi, H. (2021). Exogenous nitric oxide on morphological, biochemical and antioxidant enzyme activity on savory (*Satureja hortensis* L.) plant under cadmium stress. *Journal of the Saudi Society of Agricultural Sciences, 20(*6), 417-423.

Azizi, I, & Asadi, M. (2024). Effects of selenium on structure of the essential oil isolated from *Satureja hortensis* L. (Lamiaceae) under the cadmium stress. *International Journal of Secondary Metabolite, 11*(1), 134-143. https://doi.org/10.21448/ijsm.1349944

Azizollahi, Z., Ghaderian, S.M., & Ghotbi-Ravandi, A.A. (2019). Cadmium accumulation and its effects on physiological and biochemical characters of summer savory (*Satureja hortensis* L.). *International Journal of Phytoremediation, 21*(12), 1241-1253. https://doi.org/10.1080/15226514.2019.1619163

Bahramnezhad, Z., Ghazavi, A., Ganji, A., & Mosayebi G. (2021). Investigation on the effect of combination of *Tanacetum parthenium* and *Satureja montana* methanolic extracts on the total antioxidant capacity and serum nitric oxide. *Journal of Arak University of Medical Sciences, 24*(3), 386-397. https://doi.org/10.32598/JAMS.24.3.6196.1

Bali, A.S., Sidhu, G.P.S., & Kumar, V. (2020). Root exudates ameliorate cadmium tolerance in plants: a review. *Environmental Chemistry Letters, 18*(4), 1243-1275.

Benavides, M.P., Gallego, S.M., & Tomaro, M.L. (2005). Cadmium toxicity in plants. *Brazilian Journal of Plant Physiology, 17*, 21-34.

Esmaielpour, B., Rahmanian, M., Khorramdel, S., & Gharavi, H. (2018). Effect of organic fertilizers on nutrients content and essential oil composition of savory (*Satureja hortensis* L.). *Agritech*, *38(4),* 433-441.

Fierascu, I., Dinu-Pirvu, C.E., Fierascu, R.C., Velescu, B.S., Anuta, V., Ortan, A., & Jinga, V. (2018). Phytochemical profile and biological activities *of Satureja hortensis* L.: a review of the last decade. *Molecules, 23(*10), 2458.

Haider, F.U., Liqun, C., Coulter, J.A., Cheema, S.A., Wu, J., Zhang, R., Wenjun, M., & Farooq, M. (2021). Cadmium toxicity in plants: impacts and remediation strategies. *Ecotoxicology and Environmental Safety, 211*, 111887.

Karimi, N., Khanahmadi, M., & Soheilikhah, Z. (2013). The effect of arsenic and heavy metals on growth and metal accumulation by artichoke (*Cynara scolymus* L.) and savory (*Satureja hortensis* L.). *Iranian Journal of Plant Physiology*, *3*(3), 737-747.

Kumar, D., & Ohri, P. (2023). Say “NO” to plant stresses: unravelling the role of nitric oxide under abiotic and biotic stress. *Nitric Oxide, 130*, 36-57.

Memari-Tabrizi, E.F., Yousefpour-Dokhanieh, A., & Babashpour-Asl, M. (2021). Foliar-applied silicon nanoparticles mitigate cadmium stress through physio-chemical changes to improve growth, antioxidant capacity, and essential oil profile of summer savory (*Satureja hortensis* L.). *Plant Physiology and Biochemistry, 165*, 71-79.

Mihajilov-Krstev, T., Radnović, D., Kitić, D., Zlatković, B., Ristić, M., & Branković, S. (2009). Chemical composition and antimicrobial activity of *Satureja hortensis* L. essential oil. *Open Life Sciences, 4(*3), 411-416.

Mohtashami, S., Babalar, M., Tabrizi, L., Ghani, A., Rowshan, V., & Shokrpour, M. (2021). Essential oil constituents and variations in antioxidant compounds of dried summer savory (*Satureja hortensis* cv. *saturn*) affected by storage conditions and ammonium sulfate. *Food Science and Nutrition, 9*(9), 4986-4997. https://doi.org/10.1002/fsn3.2451

Mohtashami, S., Rowshan, V., Tabrizi, L., Babalar, M., & Ghani, A. (2018). Summer savory (*Satureja hortensis* L.) essential oil constituent oscillation at different storage conditions. *Industrial Crops and Products, 111*, 226-231. https://doi.org/10.1016/j.indcrop.2017.09.055

Mumivand, H., Babalar, M., Hadian, J., & Fakhr-Tabatabaei, M. (2011). Plant growth and essential oil content and composition of *Satureja hortensis* L. cv. *saturn* in response to calcium carbonate and nitrogen application rates. *Journal of Medicinal Plant Research, 5*(10), 1859-1866.

Najafi, F., & Khavari-Nejad, R.A. (2010). The effects of salt stress on certain physiological parameters in summer savory (*Satureja hortensis* L.) plants. *Journal of Stress Physiology and Biochemistry, 6*(1), 13-21.

Prieto, J.M., Iacopini, P., Cioni, P., & Chericoni, S., (2007). In vitro activity of the essential oils of *Origanum vulgare*, *Satureja montana* and their main constituents in peroxynitrite-induced oxidative processes. *Food Chemistry, 104*(3), 889-895.

Ribeiro, V.E., Pereira, M.P., de Castro, E.M., Corrêa, F.F., Cardoso, D.M., & Pereira, F.J. (2019). Enhanced essential oil and leaf anatomy of *Schinus molle* plants under lead contamination. *Industrial Crops and Products, 132*, 92-98. https://doi.org/10.1016/j.indcrop.2019.02.014

Şahin, F., Karaman, I., Güllüce, M., Öğütçü, H., Şengül, M., Adıgüzel, A., Öztürk, S., & Kotan, R. (2003). Evaluation of antimicrobial activities of *Satureja hortensis* L. *Journal of Ethnopharmacology, 87*(1), 61-65.

Said-Al Ahl, H.A., & Hussien, M.S. 2016. Effect of nitrogen and phosphorus application on herb and essential oil composition of *Satureja montana* L. 'carvacrol' chemotype. *Journal of Chemistry and Pharmacology Research, 8*(6), 119-28.

Selvi, S., Polat, R., Çakılcıoğlu, U., Celep, F., Dirmenci, T., & Ertuğ, Z.F (2022). An ethnobotanical review on medicinal plants of the Lamiaceae family in Turkey. *Turkish Journal of Botany, 46*(4), 283-332. https://doi.org/10.55730/1300-008X.2712

Simontacchi, M., Galatro, A., Ramos-Artuso, F., & Santa-María, G.E., (2015). Plant survival in a changing environment: the role of nitric oxide in plant responses to abiotic stress. *Frontiers in Plant Science, 6*, 977. https://doi.org/10.3389/fpls.2015.00977

Tripathi, D.K., Mishra, R.K., Singh, S., Singh, S., Vishwakarma, K., Sharma, S., Singh, V.P., Singh, P.K., Prasad, S.M., Dubey, N.K. & Pandey, A.C. (2017). Nitric oxide ameliorates zinc oxide nanoparticles phytotoxicity in wheat seedlings: implication of the ascorbate-glutathione cycle. *Frontiers in Plant Science, 8*, 1-8

Vafa, Z.N., Sirousmehr, A.R., Ghanbari, A., Khammari, I., & Falahi, N. (2015). Effects of Nano Zinc and Humic acid on quantitative and qualitative characteristics of savory *(Satureja hortensis* L.). *International Journal of Biosciences, 6(*3), 124-36.

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journal homepage: https://dergipark.org.tr/en/pub/ijsm **Research Article**

Antimicrobial and antibiofilm studies on three endemic species of *Verbascum* L. (Scrophulariaceae) in Türkiye

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Abstract: The consumption of traditional medicinal herbs has gained popularity as a viable alternative approach for addressing microbial infections or infectious structures. In this study, *Verbascum detersile, Verbascum eskisehirensis,* and *Verbascum gypsicola* endemic species belonging to the Scrophulariaceae family, spreading in Eskişehir and its surroundings, were collected from natural habitats and evaluated in terms of antimicrobial and antibiofilm activities. In biological activity studies, different concentrations of three plant extracts showed various antimicrobial and antibiofilm activities on selected standard microorganism cultures (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028, and *Candida krusei* ATCC 6258). It was observed that the selected three endemic *Verbascum* extracts had a minimum inhibitory and minimum biofilm eradication concentration value of 1250 µg/mL.

1. INTRODUCTION

Antimicrobial and antibiofilm agents are the compounds employed to inhibit the growth of bacteria or induce their elimination, while concurrently destroying the biofilm structure they form on various surfaces. Nevertheless, the efficacy of pharmaceuticals in terms of their antimicrobial or antibiofilm capabilities is constrained by the presence of resistant microorganisms. There is an urgent need for the development of novel, biologically active substances that are both environmentally friendly and devoid of toxicity to effectively combat diseases and minimize their negative consequences. The structures in question are known as microbial biofilms, which provide a conducive environment for microorganisms to thrive within a sophisticated matrix (Sánchez *et al*., 2016). Biofilm formation is considered a significant pathogenicity component in microorganisms. Plants have been widely recognized as highly promising bioactive agents. In contemporary times, there has been a notable increase in the focus on the bioactivities exhibited by plants, which have the potential to provide protection against infections or act as preventive measures.

The genus *Verbascum* L. (mullein) is one of the medicinal plants belonging to the family of Scrophulariaceae commonly known as "Sığırkuyruğu". The family of Scrophulariaceae is one of the largest plant families in dicotyledonous angiosperms with 200 genera (Gökmen, 2021).

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Verbascum genus exists predominantly in Europe, North America, and West and Central Asia (especially Anatolia). The *Verbascum* genus is represented in the world with nearly 360 species (Hunt, 2009). In Türkiye, the *Verbascum* genus consists of approximately 255 species, 200 of which are endemic (about 80% endemism rate), and it has 130 additional hybrid species. The genus exhibits a notably high endemism ratio of 80%, as seen by the presence of 196 species that are native to certain regions (Duman *et al.*, 2020). *Verbascum* species are a popular herb with medicinal uses. In traditional Turkish folk medicine, these medicinal plants are used in the treatment of expectorant, stomachache, stomach ulcer, diabetes, hemorrhoid, rheumatism, and urinary tract infection (Kargıoğlu *et al*., 2008; Mükemre *et al*., 2015). It has been reported that they are used for respiratory disorders, expectorants, stomach tonic, dyspepsia, diarrhea, diuretics, snake bites, blood clotting of women after childbirth, wound disinfection, and sedative in Iran (Ghorbani, 2005; Mohamadi *et al*., 2015). In addition, the Herbal Medicinal Products Committee (HMPC) reported that Mullein flowers (*Verbascum phlomoides*, *Verbascum thapsus*, and *Verbascum densiflorum*) can be used to soothe the throat in colds and dry coughs (EMA, 2021). In some studies, *Verbascum* has been found to have an antiviral (Escobar *et al*., 2012), antimicrobial (Senatore *et al*., 2007), enzyme inhibitory activities (Georgiev *et al*., 2011), and wound healing properties (Süntar *et al*., 2010). The medicinal benefits of *Verbascum* species are attributed to their biologically active components, including phenylethanoids, flavonoids, glycosides, neolignan, monoterpene glycosides, and saponins. (Küçük *et al*., 2016).

In Türkiye, *Verbascum detersile* Boisse & Heldr., *Verbascum gypsicola* Vural & Aydoğdu and *Verbascum eskisehirensis* Karavel., Ocak & Ekici are known as "Zinemit", "Mermer Sığırkuyruğu" and "Eski Sığırkuyruğu" respectively (Karavelioğulları *et al*., 2012). *Verbascum detersile* is in the VU category and has been reported to have a high risk of extinction. *Verbascum gypsicola* and *Verbascum eskisehirensis* species are in the CR category according to IUCN criteria and are reported as highly endangered. In this research, these three endemic species were evaluated for their antimicrobial and antibiofilm properties. These species are endemic to the Anatolia region. *V. detersile* is a biennial herb with yellow flowers. This plant prefers *Pinus* forests and rocks from sea level up to 300 m altitude and generally grows in Antalya (Saltan *et al*., 2011). *V. gypsicola* has been documented in three specific locations inside Ankara, where it thrives in very soil conditions (Vural *et al*., 1993). Additionally, it has been observed in a single locality in Eskişehir (Öztürk *et al*., 2018). *V. eskisehirensis* is a biennial herb that exclusively thrives in specific regions, including Eskişehir, Sivrihisar, Karacaören, and the Mountain of Arayit. According to Karavelioğulları *et al.* (2009), this specific plant exhibits a preference for limestone rocks and scree places. This study, therefore, aims to investigate the potential antimicrobial and antibiofilm activities of these three distinct endemic species of *Verbascum.*

2. MATERIAL and METHODS

2.1. Preparation of Plant Extracts

The aerial parts (above-ground parts) of *Verbascum* species were gathered and subsequently brought to the laboratory. Table 1 presents the geographical data about three distinct species. The collected plants were identified by Prof. Dr. Sevim Küçük (Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 26470, Tepebaşı, Eskişehir). The plant materials, which had been dried and ground into a powder, were accurately weighed and subjected to extraction using a 70% ethanol solution. This process was carried out in appropriate glass flasks. The extracts were incubated in a water bath set to room temperature with continuous agitation, and the resulting filtrates were collected by replacing the solvent every 24 hours. The procedure was sustained for three consecutive days. The extracts obtained were concentrated using a rotary evaporator and subsequently subjected to lyophilization. The desiccated extracts were stored at a temperature of +4°C following the processes of evaporation and lyophilization (Öztürk *et al*., 2019).

Table 1. Locations of the collected *Verbascum* species

Verbascum Species	Locations
V. detersile Boiss. & Heldr.	B3 Eskişehir: Alpu-Gölalan yolu, 768 m., 39°45'39.09- 30°58'22.00, 05.07.2019 (ESSE 15614)
V. gypsicola Vural & Aydoğdu	B4 Ankara: Beypazarı: Çayırhan-Beypazarı, 2 km, jipsli step, 503 m., 40°11'89.23- 31°63'89.53, 2.07.2019 (ESSE 15615)
V. eskisehirensis Karavel., Ocak & Ekici	B3 Eskişehir: Sivrihisar: Sivrihisar-Kaymaz, 1100 m., 39°26' 57.73- 31°31' 57.18, 01.06.2019 (ESSE 15616)

2.2. Determination of Antimicrobial Activity

2.2.1. *Well diffusion test*

In the test standard pathogenic microorganisms were used; *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028 and *Candida. krusei* ATCC 6258. The microorganism strains were prepared on appropriate media Mueller Hinton Agar (MHA) for bacteria and Saboroud Dextrose Agar (SDA) for yeasts. The number of microorganisms per milliliter was adjusted to $1x10^8$ cfu/mL according to the McFarland 0.5 standard. 100 µL of microorganism cultures were inoculated into suitable solid media with a Drigalski spatula. 6 mm wells were made on the medium and 25 µL of V1 (*V. detersile*), V2 (*V. eskisehirensis*), and V3 (*V. gypsicola*) was added to the wells. The plates were then incubated at 37 °C for 24 hours (Ferraro, 2000). The experiment was repeated 3 times, and the results were averaged.

2.2.2. *Broth microdilution method*

The broth microdilution method, according to the guidelines set by the Clinical and Laboratory Standards Institute (CLSI), was employed to determine the minimum inhibitory concentration (MIC) of three plant extracts (CLSI, 2002; CLSI, 2012). MIC values were determined in 96-well plates for concentrations of 5000-39.0625 µg/mL of plant extracts V1 (*V. detersile*), V2 (*V. eskisehirensis*), V3 (*V. gypsicola*). Fresh cultures of microorganisms were prepared overnight on suitable media, such as MHA for bacteria and SDA for yeasts. The pathogenic microorganisms used in this study were the same as in well diffusion method. These microorganisms were prepared following the McFarland 0.5 standard, and the concentration of microorganism colonies per milliliter was adjusted to $1x10^8$. Plant extracts were diluted in a 96-well plate and 100 µL of pathogenic microorganisms were added to them. The plates were incubated at 37 °C for 24 hours. The standard antibiotics (Ketoconazole, Chloramphenicol) 0.4-1000 µg/mL were used as a positive control group. As the negative control group, MHB and SDB medium, as well as growth control groups for microorganisms, were transferred to a 96-well plate in 2 parallels. After incubation, 20 µL of resazurin dye was added to the wells and incubated for 4 hours. After 4 hours, the results were analyzed based on the observed alterations in the wells' coloration, which spanned from a blue-green hue to a pink shade. The experiment was replicated three times, and the results were subsequently averaged.

2.3. Determination of Antibiofilm Activity

The antibiofilm activity of microorganisms was determined by the Minimum Eradication Concentration (MBEC) method (Cruz, Shah & Tammela, 2018). Biofilm-forming pathogenic microorganisms (*S. aureus* ATCC 29213, *S. epidermidis* ATCC 14990, *P. aeruginosa* ATCC 27853) were prepared according to McFarland 0.5 standard and the number of microorganisms per milliliter was adjusted to $1x10^8$ cfu/mL. 200 µL of microorganism cultures were transferred to wells of 96-well plates and incubated at 37 °C for 48 hours to the formation of biofilms. After incubation, the suspensions (200 µL) in the wells were withdrawn and the wells were washed 2 times with 0.9% NaCl solution. The concentrations (5000, 2500, 1250, 625, 312.5 µg/mL) of the plant (V1 (*V.detersile*), V2 *(V. eskisehirensis*), V3 (*V. gypsicola*)) extracts were obtained by macrodilution in MHB medium for bacteria and SDB for yeasts. Subsequently, 100 µL of plant

extracts were individually introduced into the wells, followed by incubation of the plate at a temperature of 37 °C for 24 hours. Following the incubation period, a volume of 20 µL of resazurin dye was introduced into the wells and subsequently incubated at a temperature of 37 °C for 3 hours. Following the designated waiting period, the outcomes were assessed based on the observed alterations in the wells' coloration, which might vary from a blue (or green) hue to a pink shade. MBEC values (without living cells in blue (green) color) were determined at different concentrations according to the microorganisms. The experiment was repeated three times, and the results were subsequently averaged.

3. FINDINGS

3.1. Well Diffusion Test

In the good diffusion test, it was observed that 156.25 µg/mL concentrations of the three *Verbascum* extracts did not show any antimicrobial effect against *C. albicans* and *C.krusei* yeast cells. All the *Verbascum* extracts showed antimicrobial activity against *S. aureus, S. epidermis*, and *P.aeruginosa* bacteria strains. *V. eskisehirensis* and *V. detersile* extracts demonstrated moderate antimicrobial activity when compared with amoxicillin, especially against *S. aureus* and *P.aeruginosa.* In addition to these results, *V. gypsicola* extracts showed strong antimicrobial activity with a 10 mm zone inhibition diameter as amoxicillin. The values of the zone diameters in (mm) of the extracts against the standard pathogenic microorganisms are given in Table 2.

Table 2. Values of the zone diameters (mm) formed in the well Diffusion results (V1: *V. detersile*, V2: *V. eskisehirensis*, V3: *V. gypsicola*)

Plant Extract Codes – Standard Tests Microorganisms	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*C. albicans*	*C. krusei*
V1 (156.25 µg/mL)	4±1	4±1	6±1	-*	-*
V2 (156.25 µg/mL)	10±1	6±1	6±1	-*	-*
V3 (156.25 µg/mL)	2±1	2±1	10±1	-*	-*
Amoxicillin (30 µg/mL)	4±1	32±1	10±1	-**	-**
Ketokonazol (1 mg/mL)	-**	-**	-**	14±1	30±1

* Insufficient-Low Concentration, ** Ineffective

3.2. Broth Microdilution Method

MIC values of plant extracts are given in Table 3. According to the test result, three plant extracts showed antimicrobial activity with different concentration values. The MIC values were in the range of 312.5 to 1250 µg/ml. The most prominent effect was achieved for *V. gypsicola* extract against *S. aureus* and *C. krusei*. All extracts demonstrated strong antifungal activity on *C. krusei* when compared with ketoconazole.

Table 3. MIC Values (µg/mL)

Plant Extract Codes – Standard Tests Microorganisms	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*C. albicans*	*C. krusei*
V1	1250	1250	625	1250	312.5
V2	1250	1250	625	1250	156.25
V3	625	1250	625	1250	312.5
Chloramphenicol	31.25	31.25	62.5	-**	-**
Ketoconazole	-**	-**	-**	500	500

** Ineffective

3.3. Determination of Antibiofilm Activity

Antibiofilm activity was determined by MBEC, and the results are given in Table 4. The MBEC values were in the range of 625 to 2500 µg/ml. As a result of the antibiofilm test, the biofilm structure of *S. aureus* was more sensitive to *V. eskisehirensis* and *V. detersile* extracts at the 625 µg/mL concentration. *S. epidermidis* is a prevalent pathogen with the primary pathogenic factor of creating cohesive biofilms (Knobloch *et al*., 2001). Because of this property, all *Verbascum* extracts demonstrated weak antibiofilm activity against *S. epidermidis* at the concentration of 2500 µg/mL.

Table 4. MBEC Values (µg/mL).

Plant Extract Codes – Standard Tests Microorganisms	*S. aureus*	*S. epidermidis*	*P. aeruginosa*
V1	625	2500	1250
V2	625	2500	1250
V3	1250	2500	1250

4. DISCUSSION and CONCLUSION

Certain plants belonging to the genus *Verbascum* have been employed for numerous millennia in the treatment of both internal and exterior illnesses. In the relevant literature, it has been reported that *Verbascum* species have various antimicrobial activities. In some studies, it was observed that *Verbascum* spp. was especially effective against Gram-positive and Gram-negative bacteria but did not show antifungal activity against *Candida* yeasts (Khafagi, 2002). In another study, it was commented that some *Verbascum* species showed antibacterial activity only against Gram-positive bacteria and were ineffective against Gram-negative bacteria and yeasts (Dülger *et al*., 2002). Amirnia *et al.* (2011) studied the possible antimicrobial activity of alcoholic and aqueous extracts of flowers of *V. speciosum*. Antimicrobial tests were performed by using the disc diffusion method, which included the *B. subtilis, B. cereus,* and *E. coli* bacteria strains. The extracts exhibited concentration-dependent inhibitory effects on bacterial strains. Additionally, results showed that ethanolic extract exhibited more potent activity on bacterial strains. Amirnia *et al.* (2011) reported that the highest level of activity was detected against *B. cereus*, followed by *B. subtilis*. Additionally, it was found that *E. coli* exhibited the highest level of resistance among the strains tested. Unlike these studies, both antibacterial and antifungal activities were observed in *Verbascum* species used within the scope of the research. According to the data reported by Saltan *et al.* (2011), chloroform and methanol extracts of *Verbascum detersile* showed antibacterial activity at MIC concentrations in the range of 7.5-150 mg/mL. The species on which *V. detersile* was most effective were *E. coli* ATCC 25922, *P. aeroginosa* ATCC 15442, *Streptococcus salivarius* RSHE 606, and these results were reported as 7.5 mg/mL for chloroform extract and 9.3 mg/mL for methanol extract, respectively. Öztürk *et al.* (2019) studied *V. eskisehirensis* methanol extract and stated that it has moderate antibacterial activity thanks to its flavonoid content. This is due to the verbascoside and luteolin substances in the flavonoid content. When the data obtained as a result of antimicrobial tests in this study were evaluated, antifungal activity was observed only as a result of the microdilution method. The reason for this is that the microdilution method is more sensitive than the well diffusion method and plant extracts were tested at different concentrations in the microdilution method. In the article published by Ocak *et al.* (2020), contrary to the result obtained in this study, it was reported that the methanol extract of *V. gysicola* applied by the good diffusion method had antifungal activity on *Candida albicans*. When the MIC results were evaluated, it was observed that the three plant species showed both antibacterial and antifungal activity at concentrations close to each other. In the publication of Grigorov *et al.* (2023), the antibacterial activity results obtained from the ethanol extract obtained from the flowers of *V. niveum* species are similar to the results obtained in this study. In addition to antibacterial results, antifungal values obtained

against yeasts are also similar. In the study of Göse and Hacıoğlu Doğru (2019), it was reported that ethanol extracts obtained from *V. pinnatifidum* have antibiofilm activities, especially against the biofilm structure formed by Gram-positive bacteria. This activity was proven for the first time in this study at concentrations of 2.5-10 µg/ml of the plant. In another study, Göse and Hacıoğlu Doğru (2021) reported that *V. pinnatifidum* and *V. antinori* extracts showed an inhibition activity on the biofilm structure of *B. subtilis* ATCC 6633 by 92.18% and 91.19%, respectively. Bacterial biofilms possess significant pathogenicity attributes owing to their notable resistance capabilities against chemotherapeutic agents (Grant & Hung, 2013). Bacterial biofilm formation could be controlled by a communication mechanism named quorum sensing. Hence, our study hypothesized that *Verbascum* extracts can suppress the quorum-sensing mechanism. In this study, different concentrations of the three *Verbascum* extracts showed various antimicrobial and antibiofilm activities on selected standard cultures of microorganisms. The diversity of the results of biological activity studies with *Verbascum* species is directly related to the locality where the plants were collected, ecological conditions, seasonal changes, and differences in extraction processes.

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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

Pervin Soyer: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing-original draft. **Sevim Küçük**: Methodology, Supervision, and Validation. **Yağmur Tunalı**: Supervision, and Validation.

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REFERENCES

Amirnia, R., Khoshnoud, H., Alahyary, P., Ghiyasi, M., Tajbakhsh, M., & Valizadegan, O. (2011). Antimicrobial activity of *Verbascum speciosum* against three bacteria strains. *Fresenius Environmental Bulletin*, *20*(3).

Clinical and Laboratory Standards Institute M27-A2. (2002). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Second Edition, CLSI document 2002a; 22(15).

Clinical and Laboratory Standards Institute M07-A9. (2012). Reference Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Ninth Edition, CLSI document M07-A9 2012; 29(2).

Cruz, C.D., Shah, S., & Tammela, P. (2018). Defining conditions for biofilm inhibition and eradication assays for Gram-positive clinical reference strains. *BMC microbiology*, *18*(1), 1-9. https://doi.org/10.1186/s12866-018-1321-6

Duman, H., Uzunhisarcıklı, M.E., & Özbek, F. (2020). A new species of *Verbascum* (Scrophulariaceae) from western Türkiye. *Botany Letters*, *167*(4), 409–416. https://doi.org/10.1080/23818107.2020.1796782

Dülger, B., Kirmizi, S., Arslan, H., & Güleryüz, G. (2002). Antimicrobial Activity of Three Endemic *Verbascum* Species. *Pharmaceutical Biology*, *40*(8), 587-589. https://doi.org/10.1076/phbi.40.8.587.14657

EMA. (2021). *Verbasci flos - European Medicines Agency*. European Medicines Agency. https://www.ema.europa.eu/en/medicines/herbal/verbasci-flos

Escobar, F.M., Sabini, M.C., Zanon, S.M., Tonn, C.E., & Sabini, L.I. (2012). Antiviral effect and mode of action of methanolic extract of *Verbascum thapsus* L. on pseudorabies virus (strain RC/79). *Natural Product Research*, *26*(17), 1621-1625. https://doi.org/10.1080/14786419.2011.576394

Ferraro, M.J. (2000). *Performance Standards for Antimicrobial Disk Susceptibility Tests*.

Georgiev, M., Alipieva, K., Orhan, I., Abrashev, R., Denev, P., & Angelova, M. (2011). Antioxidant and cholinesterases inhibitory activities of *Verbascum xanthophoeniceum* Griseb. and its phenylethanoid glycosides. *Food Chemistry*, *128*(1), 100–105. https://doi.org/10.1016/j.foodchem.2011.02.083

Ghorbani, A. (2005). Studies on pharmaceutical ethnobotany in the region of Turkmen Sahra, north of Iran. *Journal of Ethnopharmacology*, *102*(1), 58-68. https://doi.org/10.1016/j.jep.2005.05.035

Gökmen, A. (2021). Phytochemical and Biological Investigations on *Verbascum bugulıfolıum* L. [Doctoral dissertation]. Yeditepe University.

Göse, M., & Doğru, N.H. (2019). Antibiofilm activity of *Verbascum pinnatifidum* Vahl. ethanolic extract. Biologica Nyssana, *10*(2). https://doi.org/10.5281/zenodo.3600197

Göse, M., & Hacıoğlu, N. (2021). Bioactive compounds, antimicrobial and antibiofilm activity of two *Verbascum* species. Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi, *24*(3), 479-487. https://doi.org/10.18016/ksutarimdoga.v24i60916.750034

Grant, S.S., & Hung, D.T. (2013). Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. *Virulence*, *4*(4), 273–283. https://doi.org/10.4161/viru.23987

Grigorov, M., Pavlović, D., Mladenović, A.S., Tasić, K.M., & Ilić, D. (2023). In vitro antimicrobial activity of different *Verbascum niveum* extracts. Acta facultatis medicae Naissensis, *40*(2), 193-198. https://doi.org/10.5937/afmnai40-40582

Hunt, C. (2009). Mabberley's Plant-Book: A Portable Dictionary of Plants, their Classifications and Uses (3rd edition)2009175David. J. Mabberley. Mabberley's Plant-Book: A Portable Dictionary of Plants, their Classifications and Uses (3rd edition). Cambridge: Cambridge University Press 2008. xviii+1021 pp., ISBN: 978 0 521 82071 4 £50, $90. *Reference Reviews*, *23*(4), 35–36. https://doi.org/10.1108/09504120910958511

Karavelioğulları, A.F., Ocak, A., Ekici, M., & Cabi, E. (2009). *Verbascum eskisehirensis* sp. nov. (Scrophulariaceae) from central Anatolia, Türkiye. *Nordic Journal of Botany*, *27*(3), 222–227. https://doi.org/10.1111/j.1756-1051.2008.00250.x

Karavelioğulları, F.A. *Verbascum* L. In: Güner, A. Aslan, S. Ekim, T. Vural, M., & Babaç, M. T. (eds.). (2012). Türkiye Bitkileri Listesi (Damarlı Bitkiler). Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını, İstanbul, pp. 850-870.

Kargıoğlu, M., Cenkci, S., Serteser, A., Evliyaoğlu, N., Konuk, M., Kök, M.A., & Bağcı, Y. (2008). An Ethnobotanical Survey of Inner-West Anatolia, Türkiye. *Human Ecology*, *36*(5), 763–777. https://doi.org/10.1007/s10745-008-9198-x

Khafagi, I.K. (2002). Screening in vitro cultures of some Sinai medicinal plants for their antibiotic activity. *Egyptian Journal of Microbiology (Egypt)*.

Knobloch, J.K.M., Bartscht, K., Sabottke, A., Rohde, H., Feucht, H.H., & Mack, D. (2001). Biofilm formation by *Staphylococcus epidermidis* depends on functional RsbU, an activator of the sigB operon: differential activation mechanisms due to ethanol and salt stress. *Journal of Bacteriology*, *183*(8), 2624-2633. https://doi.org/10.1128/jb.183.8.2624-2633.2001

Küçük, S., Özdemir, F., İşcan, G., & İncesu, Z. (2016). Determination of Cytotoxic and Anticandidal Activities of Three *Verbascum* L. Species from Türkiye: *V. cheiranthifolium*

Boiss. var. asperulum (Boiss.) Murb. Monorg., *V. pycnostachyum* Boiss. & Heldr and V. orgyale Boiss. & Heldr. *Turkish Journal of Pharmaceutical Sciences*, *13*(3), 318–322. https://doi.org/10.4274/tjps.2016.06

Mohamadi, N., Sharififar, F., Koohpayeh, A., & Daneshpajouh, M. (2015). Traditional and Ethnobotanical uses of medicinal plants by ancient populations in Khabr and Rouchon of Iran. *Journal of Applied Pharmaceutical Science*, 101-107. https://doi.org/10.7324/japs.2015.501117

Mükemre, M., Behçet, L., & Çakılcıoğlu, U. (2015, May). Ethnobotanical study on medicinal plants in villages of Çatak (Van-Türkiye). *Journal of Ethnopharmacology*, *166*, 361–374. https://doi.org/10.1016/j.jep.2015.03.040

Ocak, E., İnci, Ş., Öztürk, D., Şafak, S. A., Özdeniz, E., Kırbağ, S., ..., & Kurt, L. (2021). Antimicrobial activities of some narrow endemic gypsopyhte. İstanbul Journal of Pharmacy, *51*(1), 118-122. https://doi.org/10.26650/IstanbulJPharm.2020.0084

Öztürk, D., Sezer, O., Koyuncu, O., & Ocak, A. (2018). Flora of gypsiferous and marl soils in Eskisehir (Türkiye). *Biological Diversity and Conservation*, *11*(2), 137-15.

Öztürk, G., Ağalar, H., Yildiz, G., Göger, F., & Kırımer, N. (2019). Biological activities and luteolin derivatives of *Verbascum eskisehirensis* Karavel., Ocak & Ekici. Journal of Research in Pharmacy, *23*(3). https://doi.org/10.12991/jrp.2019.160

Saltan, F.Z., Sokmen, M., Akin, M., Saracoglu, H.T., Gokturk, R.S., Ahmad, M., ..., & Shah, M.R. (2011). Antimicrobial and antioxidant activities of phenolic compound extracted from new *Verbascum* species growing in Türkiye. *Journal of the Chemical Society of Pakistan*, *33*(5),764-771. https://inis.iaea.org/search/search.aspx?orig_q=RN:43027999

Sánchez, E., Rivas Morales, C., Castillo, S., Leos-Rivas, C., García-Becerra, L., & Ortiz Martínez, D. M. (2016). Antibacterial and Antibiofilm Activity of Methanolic Plant Extracts against Nosocomial Microorganisms. *Evidence-Based Complementary and Alternative Medicine*, *2016*, 1–8. https://doi.org/10.1155/2016/1572697

Senatore, F., Rigano, D., Formisano, C., Grassia, A., Basile, A., & Sorbo, S. (2007). Phytogrowth-inhibitory and antibacterial activity of *Verbascum sinuatum*. *Fitoterapia*, *78*(3), 244–247. https://doi.org/10.1016/j.fitote.2006.11.010

Süntar, I., Tatlı, I.I., Küpeli Akkol, E., Keleş, H., Kahraman, I., & Akdemir, Z. (2010). An ethnopharmacological study on *Verbascum* species: From conventional wound healing use to scientific verification. *Journal of Ethnopharmacology*, *132*(2), 408-413. https://doi.org/10.1016/j.jep.2010.08.004

Vural, M., & Aydoğdu, M. (1993). A new species from central Anatolia *Verbascum gypsicola* (Scrophulariaceae). *The Karaca Arboretum Magazine*, *2*(2), 75-78.

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Research Article

Cytotoxic effect of *Eleutherococcus trifoliatus* (L.) S.Y. Hu stem bark extracts on Gastric, Lung, and Hepatocellular cancer cells

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Abstract: *Eleutherococcus trifoliatus* is a popular medicinal species used in Asian folk medicine with many uses in treating human diseases. Scientific research on this plant has been limited, but some scientific publications have described this herb's anti-inflammatory, antibacterial, antioxidant, and anti-cancer activities. Although it is a frequently used part of traditional medicine, current scientific evidence on the biological activities of *E. trifoliatus* stem bark is still lacking. By performing chemical reactions, free radical scavenging experiments, toxicity experiments on brine shrimp, and cytotoxicity tests using MTT dye, the study has shown the diversity in the metabolic composition of *E. trifoliatus* stem bark as well as the antioxidant capacity and safety of the total extract. The anticancer effect of the total extract was investigated and indicated the dramatical ability to inhibit the cell growth of liver cancer HCC-J5 cells (IC50 = 19.35 ± 4.89 µg/mL), lung cancer cells A549 (IC50 = 5.34 ± 1.62 µg/mL) and gastric cancer cells AGS (IC50 = 0.22 ± 0.20 µg/mL); the selectivity in effects was also observed. The chloroform fraction had the most potential to be further exploited in the direction of inhibiting cancer cells.

1. INTRODUCTION

Eleutherococcus trifoliatus (L.) S.Y. Hu (synonym:*Acanthopanax trifoliatus* (L.) Voss.) is a small tree with medium-high (below 7 meters), robust branching, and thorning (Loi, 2004). Leaves are triangular in shape, like duck feet, with staggered distribution, and leaf veins with spines (Loi, 2004). Flowers develop at the tip of the branch with more than 3 crowns. Berried fruits are in a flat sphere shape, in black, harboring 2 seeds. Essential oils can be extracted from the whole plant with fragrance (Loi, 2004). This plant is mainly distributed in areas of Asia such as Korea, China, Far East Russia, Vietnam, and Japan. This plant has been discovered in Vietnam in Cao Bang, Lao Cai, Lang Son, Lai Chau, Son La, Hoa Binh, Thanh Hoa, Nghe An, Quang Nam, Quang Ngai, and Kom Tum. This plant is exploited in folk medicine as a medicinal herb to treat human diseases (Huyen *et al.*, 2006; Loi, 2004). Traditional herbalists believe that the root bark and stem bark are the most pharmacologically practical parts of *E. trifoliatus* and

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should be harvested in the summer (Huyen *et al.*, 2006; Loi, 2004). The medicinal powder has a bitter taste and is used in treating bone and joint diseases, inflammation, and erectile dysfunction (Loi, 2004; Thu & Tuan, 2013). In modern medicine, this medicinal herb is used for sedation, anti-inflammation, and dilation of blood vessel walls, thereby lowering blood pressure and stopping coughing and expectoration (Loi, 2004; Thu & Tuan, 2013).

Despite its widespread application, scientific research on *E. trifoliatus* has still been quite vague, preventing the exploitation of this plant in modern medicine. Thus far, scientific evidence has shown several bio-activities of *E. trifoliatus*, such as the antioxidant effect (Sithisarn & Jarikasem, 2009); anti-inflammatory on RAW264.7 cells and BALB/c mice (Chen *et al.*, 2021; Chien *et al.*, 2015; Thu & Tuan, 2013); antibacterial impact tested on *Salmonella, Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus albicans*, and *Micrococcus luteus* (Chen *et al.*, 2021); analgesic effects were observed on rats (Chen *et al.*, 2021); hemostatic effects evaluated on New Zealand white rabbits (Chen *et al.*, 2021); type 2 diabetic treatment investigated on C57BL/6 mice (Lin *et al.*, 2023). *E. trifoliatus*'s antitumor studies have also been conducted on PC-3, SF-268, MCF-7, HepG2, and NCI-H460 cells; several toxicity pathways have been described (Li *et al.*, 2016; H.-C. Wang *et al.*, 2014; H. Q. Wang *et al.*, 2014). However, research on the herb's stem bark has been still lacking. This work aimed to demystify the anti-proliferation of the *E. trifoliatus* stem bark total and fractional extracts on lung carcinoma, gastric adenocarcinoma, and hepatocellular carcinoma cells, and describe the ingredient background and antioxidant capacity of *E. trifoliatus* stem bark.

2. MATERIAL and METHODS

2.1. Extract Preparation

2.1.1. *Plant sample and extraction*

Eleutherococcus trifoliatus stem bark was derived from An Giang Province, Vietnam, with voucher 2021NCBG-AG01. The sample was dried after double washing with distilled water. The dried sample was ground into a fine powder and macerated with absolute methanol at 1:3 (weight/volume). After five days of maceration with the filtrate collection every 24 hours, the total stem bark extract (abbreviated as ET) was obtained by solvent removal. For the fractional extraction, the crude extract was dissolved with chloroform to obtain the chloroform fraction. The insoluble extract was further dissolved with ethyl acetate to obtain ethyl acetate fraction. The procedure continued with methanol and water. The results were 4 different fractions dried using the Soxhlet system, including chloroform (extract abbreviated as ETC), ethyl acetate (extract abbreviated as ETE), methanol (extract abbreviated as ETM), and water (extract abbreviated as ETW) extracts. The droughty total and fractional extracts were weighted and dissolved with DMSO (Dimethyl sulfoxide, Sigma-Aldrich, USA) to achieve the stock solution of 400 µg/mL for crude extract and 100 µg/mL for fractional extracts.

2.1.2. *Phytochemical screening*

In order to optimize the detection of plant compounds, medicinal powders were soxhleted with three solvents of different polarities as described by Ciulei (Ciulei, 1993). A mass of 30 grams of the herbal was wrapped with filter paper and extracted using the Soxhlet system with diethyl ether, ethanol, and water, respectively. The chemical reactions were performed for phytochemicals detection.

2.2. Antioxidant Effect of Total Extract

The free radical scavenging capacity of the ET was determined by performing the DPPH (2 2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, USA) and ABTS (2,2′-Azinobis-(3-Ethylbenzthiazolin-6-Sulfonic Acid), Sigma-Aldrich, USA) assays. Active ABTS solution was formed by the reaction of 2.6 mM ABTS with 7.4 mM potassium peroxydisulfate [$K_2S_2O_8$] (Sigma-Aldrich, USA) for 16 hours in the dark. The active ABTS solution was adjusted with methanol to achieve the final solution with an absorbance of 1.00 ± 0.02 at 734 nm. The extract

at different concentrations was reacted to DPPH (with a ratio of 1:1) and active ABTS (with a ratio of 1:5). After a 30-minute incubation; the mixtures measured the absorbance at 517 nm and 734 nm for DPPH assay and at 734 nm for ABTS. Nonlinear regressions computed the EC50 (half maximal effective concentration) values of the ET on DPPH and ABTS radicals.

2.3. Toxicity on Brine Shrimp

Brine shrimp cysts (*Artemia nauplii*) were hatched in an incubator with continuous light and aeration. A volume of 2 mL saline containing thirty individual shrimps was divided into separate cups. The ET extract at different concentrations ranging from 0 to 100 µg/mL was supplemented into larvae cups. The DMSO equivalent to the amount added in the most concentrated treatment was set as a negative control. After 24 hours of treatment, the dead shrimps, which showed no movement for at least 10 seconds, were counted and computed for the survival proportion (Santos Filipe *et al.*, 2022).

2.4. Cytotoxicity Evaluation

2.4.1. *Cell lines and cell culturing*

Lung carcinoma cells A549 (ATCC, USA), gastric adenocarcinoma AGS (ATCC, USA), and hepatocellular carcinoma HCC-J5 (Cell Culture Center of the National Taiwan University, Taipei, Taiwan) were used to investigate the anticancer impact of *Eleutherococcus trifoliatus* stem bark extracts. The non-cancerous cells, fibroblast BJ (ATCC, USA), were used as control. Cells were cultured in Roswell Park Memorial Institute (RPMI, Thermo Scientific, USA) 1640 Medium, added 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, USA) and 1% Penicillin-streptomycin (Sigma-Aldrich, USA). As 80% of the culture surface was occupied by cells, the cells were sub-cultured with the initial density of 10^5 cells/mL.

2.4.2. *Cytotoxicity assay*

Cells were seeded at 10^5 cells/mL density into 96-well plates. After 24 hours, the medium harboring the extract at different concentrations with homologous volumes was added to each well. The treatment was conducted for 72 hours. The cell viability was calculated by MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), Sigma-Aldrich, USA) reagent; the crystal dissolution solution was measured the absorbance at 490 nm. Nonlinear regression indicated the half-maximal inhibitory concentration (IC_{50}) values of the extracts on tested cells.

2.4.3. *Selective index*

The selective index of the ET was indicated by dividing the IC_{50} value of the ET on BJ cells by the one on cancer cells.

2.5. Data Computation

Data collection occurred at least three independent times. Documents were analyzed using Excel 365 (Microsoft, USA) and GraphPad Prism version 9.0.0 (GraphPad Software, USA). Nonlinear regression was performed according to the model Y=100/(1+10^((LogIC50-X)*HillSlope))) to deduce the IC50 and EC50 values. Comparative statements were determined from the results of ANOVA one-way analysis combined with the Tukey post-hoc test and Student T-test with an Alpha significance level of 5%. The symbol notes (*), (**), (***), and (****) stood for the *p*-value below 0.033, 0.0021, 0.0002, and 0.0001, respectively.

3. FINDINGS

3.1. Phytochemical Description

The chemical reactions exhibited the presence of polyphenol, flavonoid, alkaloid, saponin, anthocyanin, reducing sugar, cardiac glycoside, and steroid compounds (Table 1).

Table 1. Screening for phytochemicals in *E. trifoliatus* stem bark.

Compounds	Reactions	Extracts		
		Diethyl ether	Ethanol	Water
Alkaloid (Jha *et al.*, 2012)	Mayer Wagner	+ +	+ +	+ +
Flavonoid (Ly *et al.*, 2019)	Alkalize with 10% NaOH Acidified with concentrated H2SO4	+ +	+ +	- -
Steroid (Nath *et al.*, 1946)	Liebermann – Burchard		+	+
Polyphenol (MacWilliam & Wenn, 1972)	Reducing $FeCl_3$		+	+
Anthocyanin (Ly *et al.*, 2019)	Reddening induced by HCl 10% Cyanotion induced bt NaOH 10%		-	-
Saponin (Edeoga, 2005)	Foam formation			+
Cardiac glycoside (Mujeeb *et al.*, 2014)	Keller-Kiliani		+	-
Reducing sugar (Ayoola *et al.*, 2008)	Fehling			+
Organic acid (Thi *et al.*, 2023)	Na_2CO_3		-	-
Polyuronid (Thi *et al.*, 2023)	Flocculation		-	-

Presence (+); absence (-); empty cells stand for no need for detection.

3.2. Antioxidant Effect of The Total Extract

As shown in Figure 1, the radical scavenging ability of the ET was described. The EC50 (µg/mL) values were demystified at 339.50 ± 48.60 and 138.10 ± 24.70 for DPPH and ABTS. The *t*-test one-tail result showed the effect of the ET on ABTS was greater than on DPPH, P-value < 0.0379. The vitamin C at the concentration of 50 µg/mL illustrated to scavenge 87.14 ± 0.24 % DPPH and 99.66 ± 0.60 ABTS; the effect of 100 µg/mL one was 89.94 ± 0.24 % and 100 ± 0.02 % for DPPH and ABTS.

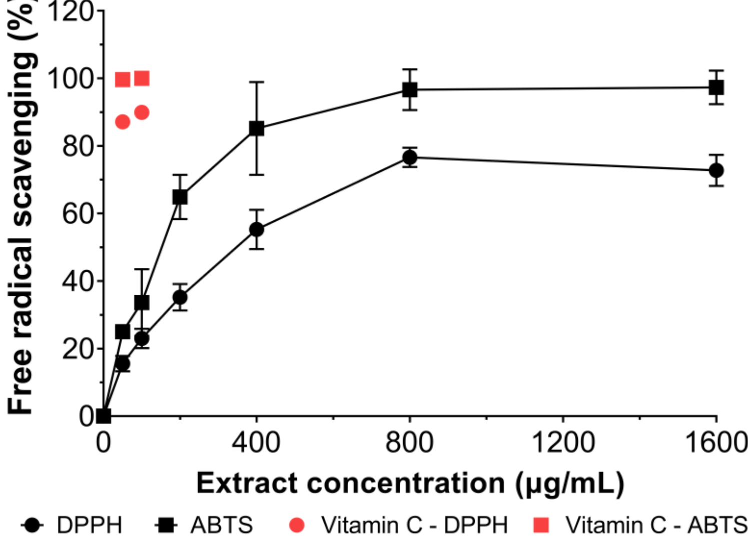

Figure 1. The free radical scavenging of the *E. trifoliatus* stem bark total extract evaluated by DPPH and ABTS assays.

3.2. Toxicity on Brine Shrimp

The ET extract showed no negative impact on the vital of *Artemia nauplii* after 24 hours of treatment, p-value = 0.2127 (Figure 2). There were no statistical differences between the experiment with DMSO and negative control, p-value = 0.1854.

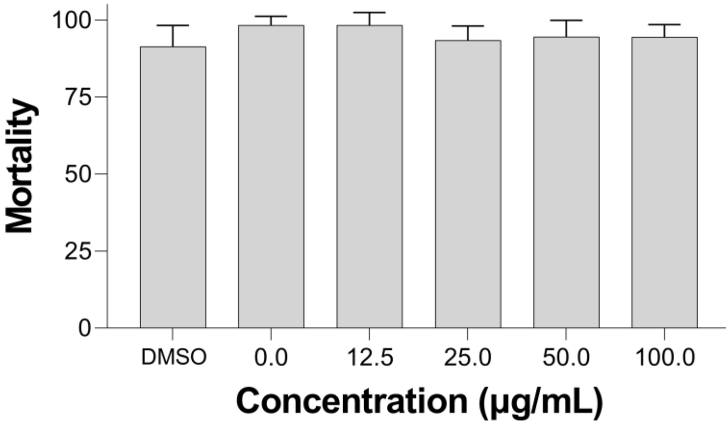

Figure 2. The toxic impact of *E. trifoliatus* stem bark total extract on brine shrimp.

3.4. Anticancer Effect of the ET on Selective Cancer Cells

The anticancer effect of ET results were shown in Figure 3. The influence was illustrated in a dose-dependent manner (Figure 3A). Non-linear regression analysis determined the IC_{50} values of ET on hepatocellular carcinoma (HCC-J5), lung carcinoma (A549), and gastric adenocarcinoma (AGS) at 19.35 ± 4.89 µg/mL, 5.34 ± 1.62 µg/mL, and 0.22 ± 0.20 µg/mL, respectively. The impact of ET on the three cell lines was statistically significant, p-value < 0.0001. The lethality of ET was recorded strongest on AGS and weakest on HCC-J5 (Figure 3B).

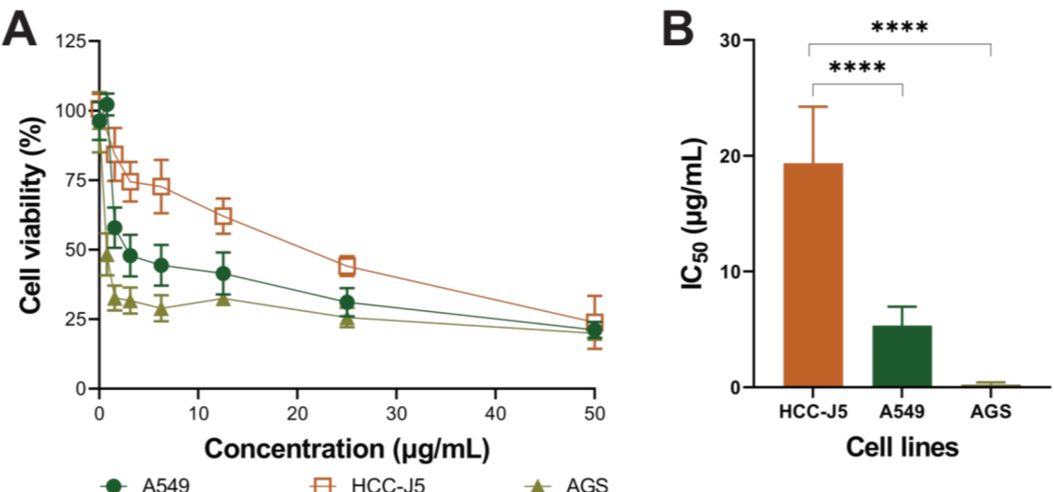

Figure 3. The cytotoxic effect of *E. trifoliatus* stem bark total extract on evaluated cancer cells (A), and the IC values of the impact (B).

3.5. ET Retards the Growth of Fibroblast

The impact of ET on BJ cells was depicted in Figure 4A. The IC_{50} of the extract on the fibroblast was calculated at 21.33 ± 6.97 µg/mL. Based on the IC_{50} values, the SI values were computed at 1.10 ± 0.28, 3.99 ± 1.21, and 95.39 ± 31.17 for HCC-J5, A549, and AGS. There is a statistically significant difference in the selectivity of ET's effects on AGS cells compared to HCC-J5 (p-value = 0.0017) and A549 (p-value = 0.0019).

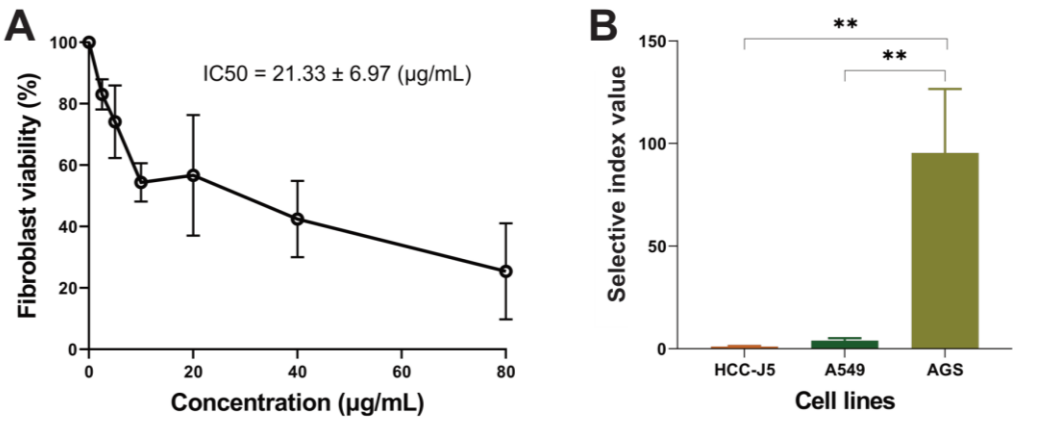

Figure 4. The inhibitory of *E. trifoliatus* stem bark total extract on fibroblast (A), and the selective index in anticancer capacity of the extract (B).

3.6. The Anticancer Effect of *E. trifoliatus* Fractions

The inhibitory of four fractional extracts of *E. trifoliatus* stem bark was illustrated in Figure 5. The impact was observed to be in a dose-dependent manner. The IC_{50} values of the fraction on tested cells are shown in Figure 6.

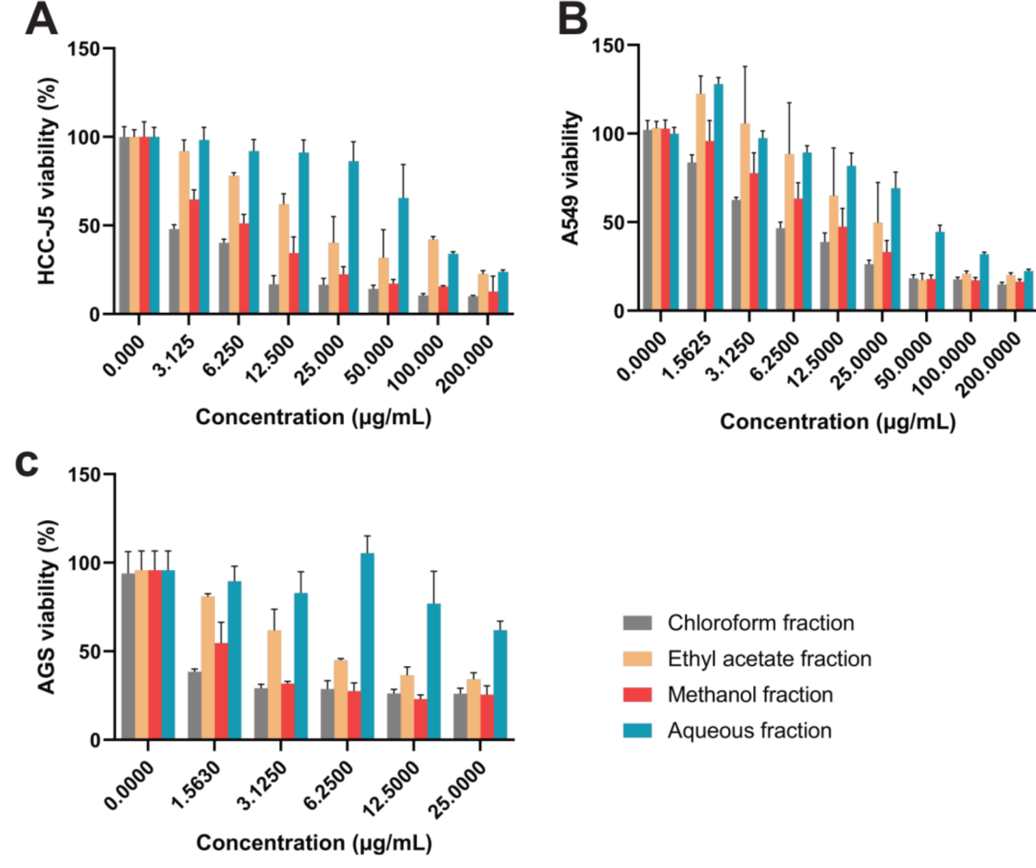

Figure 5. The cytotoxic impact of the fractional extract of *E. trifoliatus* stem bark on HCC-J5 (A), A549 (B), and AGS (C).

The expressed the IC_{50} values on HCC-J5, A549, and AGS were 2.22 ± 0.84 μg/mL, 7.10 ± 1.13 μg/mL, and 0.08 ± 0.50 μg/mL for chloroform fraction; 23.92 ± 5.56 μg/mL, 22.84 ± 5.94 μg/mL, and 6.71 ± 1.65 μg/mL for ethyl acetate fraction; 5.84 ± 1.35 μg/mL, 12.21 ± 2.03 μg/mL, and 1.28 ± 1.00 μg/mL for methanol fraction; 75.62 ± 10.61 μg/mL, 48.01 ± 7.07 μg/mL, and 34.69 ± 11.88 μg/mL for aqueous fraction. The effects of chloroform and methanol

fractions were the most effective on the three cell lines tested when compared with water and ethyl acetate fractions, p-value < 0.0001.

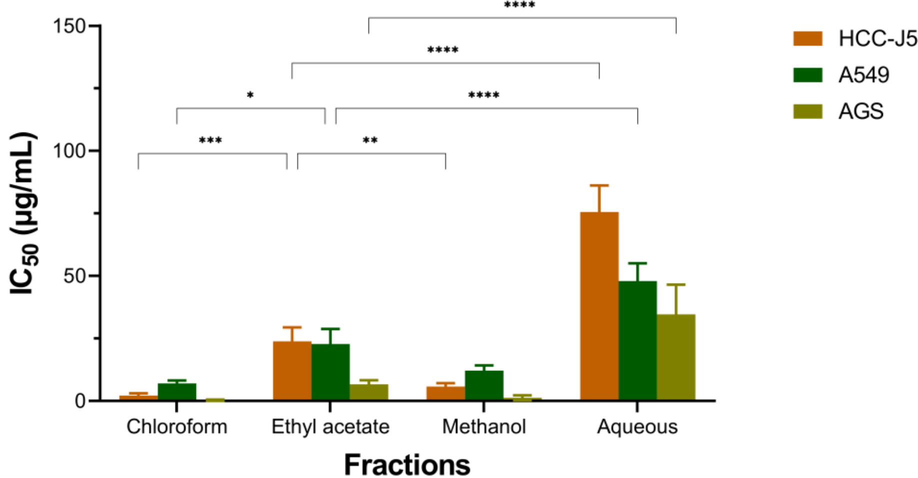

Figure 6. The IC_{50} values of the fractional extracts on evaluated cancer cells.

4. DISCUSSION and CONCLUSION

E. trifoliatus stem bark has been widely used in folk remedies in Asia; however, scientific information about this medicinal herb has not been fully described (Wiart, 2007). The presence of secondary metabolites is an important sign in predicting the bio-activity of plant extracts (Baumli *et al.*, 2023). Investigations on the phytochemical composition of *E. trifoliatus* stem bark have been still limited. The diversity in ingredients of the stem bark was described as suitable with previous reports. Some studies showed an abundance of phenolic compounds (0.65 ± 0.06 mg/g), flavonoids (0.60 ± 0.06 mg/g), and saponin (23-30 mg/g) in this plant (Chen *et al.*, 2021; Dedvisitsakul & Watla-Iad, 2022). Due to its aromatic scent, many previous studies have focused on analyzing its terpene-derived compounds, such as α-pinene, sabinene, terpinen-4-ol, β-pinene, and *p*-cymene (Muselli *et al.*, 1999; Phuong *et al.*, 2006). In the leaves, various vitamins were found, including vitamin A (428.47 ± 3.00 µg/100g), vitamin B1 (0.41 ± 0.01 µg/100g), vitamin B2 (0.17±0.00 µg/100g), vitamin C (11.95±0.86 µg/100g) (Ganogpichayagrai & Suksaard, 2020). The antioxidant bioactive of the ET could be predicted due to the presence of relevant secondary compounds.

In this study, the capacity to trap free radicals reflected the antioxidant ability of the ET extract. As the ET concentration increased, the rate of free radicals collected increased. The antioxidant impact of the *E. trifoliatus* leaf extract was recorded by using oxygen radical absorbance capacity (9057.29 ± 43.08 µmol) and ferric reducing antioxidant power (1230.88 ± 19.51 µmol), and the whole plant effect on DPPH free radical scavenging activity was 0.93 ± 0.09 mg L-ascorbic acid equivalent per gram extract (Dedvisitsakul & Watla-Iad, 2022; Ganogpichayagrai & Suksaard, 2020). The ET extract was considered to have a weak antioxidant effect, because the EC50 values were greater than 100 µg/mL (Blois, 1958). Radical neutralization has been reported to come from electron donation by natural compounds such as phenolic compounds (Lobo *et al.*, 2010). Various scientific evidence has proven the relationship between antioxidants and their bio-effect (Kancheva & Kasaikina, 2013). The presence of secondary metabolites and antioxidant capacity gave predictions about the activities of this plant.

The safety of plant extracts has an impact on their application (Ferraz *et al.*, 2022). For the purpose of toxicity determination, the brine shrimp lethality assay, which is widely used for the preliminary cytotoxicity assay of plant extract, was conducted (Harwig & Scott, 1971). The lethality of the ET was unremarkable on *A. nauplii* viability; the DMSO was also ineffective on larvae. The survival rate of shrimp in experiments always remained above 95%, and there was

no statistically significant difference in the impact of ET concentrations on larval vitality. The median lethal dose (LD50) value was considered to be greater than the value of 100 µg/mL which was classified as non-active for the crude extract, for it was not demystified during the evaluation (Muhammed *et al.*, 2021; Rieser *et al.*, 1996). In the report elsewhere, the protective ability of *E. trifoliatus* extract investigated on the liver of rats with liquor overdose showed that the extract could induce liver cell degeneration (Li *et al.*, 2021). The low toxicity facilitates ET extract in medical applications.

Notably, the ability of *E. trifoliatus* to inhibit cancer cell growth has been investigated. The results illustrated that the inhibition of cell proliferation by ET was in a dose-dependent manner. In the concentration of 50 µg/mL, the ET retarded around 75% of cell proliferation in all cell lines. The non-linear regression indicated the IC50 values of ET on HCC-J5, A549, and AGS were lower than the value of 20 µg/mL creating a boundary to classify the strong effective plant extract in cytotoxicity, in which, the effect on AGS was weighed in as excellent cytotoxic for a total extract (Indrayanto *et al.*, 2021). The impact of ET on AGS was more effective than the other two cell lines. Thus far, several studies have investigated the effects of this plant extract on cancer. PC-3 cells were observed to be inhibited under the leaf extract via interruption of the NF-kB, Erk1/2, Akt pathway (H. Q. Wang *et al.*, 2014). *E. trifoliatus*-derived terpenoids was determined as SF-268, MCF-7, HepG2 and NCI-H460 anti-growth elements (Li *et al.*, 2016). Taiwanin E isolated from *E. trifoliatus* was demonstrated as a pRB inhibitor in MCF-7 treatment (H.-C. Wang *et al.*, 2014). Moreover, the lethality impact of the ET was also investigated on fibroblast BJ with the IC50 of 21.33 ± 6.97 µg/mL leading to the classification of the effect as moderate cytotoxic. By dividing the IC50 value of ET on BJ cells by the test cancer cells, the SI values were indicated. The selectivity of an extract with an SI value less than one should be considered against its use as an herbal drug (Indrayanto *et al.*, 2021; Nogueira & Rosário, 2010). Therefore, the selectivity in the effect of ET on HCC-J5 (SI = 1.10 ± 0.28) was considered unexploitable. On the contrary, the SI values of ET on A549 and AGS exhibited the potential to exploit this medicinal plant as a source of raw materials for future treatment research. The effect of ET on A549 (SI = 3.99 ± 1.21) was classified as a prospective anti-cancer, while the impact on ASG (SI = 95.39 ± 31.17) was elected as a highly potential herb (Indrayanto *et al.*, 2021; Peña-Morán *et al.*, 2016; Weerapreeyakul *et al.*, 2012). The cytotoxic influence of *E. trifoliatus* stem bark extract on liver cancer, lung cancer, and stomach cancer was discussed for the first time in this study.

Chloroform and methanol fractions were recorded to be more cytotoxic than ethyl acetate and aqueous fractions. The inhibition induced by ETC was astounding with the IC50 on three cell lines under 10 µg/mL; the effect on AGS was most substantial with IC50 = 0.0782 + 0.50 µg/mL. The impact of ETM was also classified as a potential extract with strong cytotoxic; the ETM's IC50 was below 10 µg/mL for the one on HCC-J5 and below 2 µg/mL for AGS. The effects of the fractionated extracts were observed to be divided into two groups when compared with the effects of the total extract, including those with better inhibitory activity and those with poorer inhibitory activity. Compared to the ET, the anti-proliferated effect of ETM and ETC increased on HCC-J5; the effect of ETC augmented on AGS. The chloroform extract fraction was also shown to be effective in cytotoxicity observed in some previous studies compared to other fractions (Einafshar *et al.*, 2024; Li *et al.*, 2015; Zhang *et al.*, 2020). It could be concluded that chloroform was the effective solvent for extracting the compounds inducing cytotoxicity, and more in-depth studies are needed to be carried out to clarify it.

In conclusion, *E. trifoliatus* stem bark extract expressed a weak antioxidant effect, but the cytotoxic impact was remarkable. The inhibited impact was first recorded on lung carcinoma cells A549, gastric adenocarcinoma cells AGS, and hepatocellular carcinoma cells HCC-J5. Additionally, the tremendously selective impact of the extract was on AGS that needed to be further researched. The safety of the total extract was proven via toxicity assay. The effect was more powerful in chloroform fractionalization.

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

Nguyen Trung Quan and **Hoang Thanh Chi** designed and performed the experiments. **Nguyen Trung Quan** analyzed the data. **Bui Thi Kim Ly** wrote the original draft. All authors read, edited, and approved the final manuscript.

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REFERENCES

Ayoola, G., Coker, H., Adesegun, S., Adepoju-Bello, A., Obaweya, K., Ezennia, E., & Atangbayila, T. (2008). Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. *Tropical Journal of Pharmaceutical Research,* 7(3), 7. https://doi.org/10.4314/tjpr.v7i3.14686

Baumli, J., Antal, N., Casoni, D., & Cimpoiu, C. (2023). Use of Secondary Metabolites Profiling and Antioxidant Activity to Unravel the Differences between Two Species of Nettle. *Plants*, *12*(18), 3233. https://www.mdpi.com/2223-7747/12/18/3233

Blois, M.S. (1958). Antioxidant Determinations by the Use of a Stable Free Radical. *Nature*, *181*(4617), 1199-1200. https://doi.org/10.1038/1811199a0

Ciulei, I. (1993). *Plante medicinale , fitochimie si fitoterapie.* (Vol. II). Editura medicală.

Chen, Z., Cheng, S., Lin, H., Wu, W., Liang, L., Chen, X., . . ., Zhang, K. (2021). Antibacterial, anti-inflammatory, analgesic, and hemostatic activities of *Acanthopanax trifoliatus* (L.) merr. *Food Science & Nutrition*, *9*(4), 2191-2202. https://doi.org/10.1002/fsn3.2190

Chien, T.-M., Hsieh, P.-C., Huang, S.-S., Deng, J.-S., Ho, Y.-L., Chang, Y.-S., & Huang, G.-J. (2015). *Acanthopanax trifoliatus* inhibits lipopolysaccharide-induced inflammatory response in vitro and in vivo. *The Kaohsiung Journal of Medical Sciences, 31*(10), 499-509. https://doi.org/10.1016/j.kjms.2015.07.007

Dedvisitsakul, P., & Watla-Iad, K. (2022). Antioxidant activity and antidiabetic activities of Northern Thai indigenous edible plant extracts and their phytochemical constituents. *Heliyon*, *8*(9), e10740. https://doi.org/10.1016/j.heliyon.2022.e10740

Edeoga, H.O., Okwu, D.E. & Mbaebie, B.O. (2005). Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology. *African Journal of Biotechnology*, 4(7), 685-688.

Einafshar, E., Mobasheri, L., Hasanpour, M., Rashidi, R., & Ghorbani, A. (2024). Pro-apoptotic effect of chloroform fraction of *Moraea sisyrinchium* bulb against glioblastoma cells. *Biomedicine & Pharmacotherapy, 170*, 115931. https://doi.org/10.1016/j.biopha.2023.115931

Ferraz, C.A., Pastorinho, M.R., Palmeira-de-Oliveira, A., & Sousa, A.C.A. (2022). Ecotoxicity of plant extracts and essential oils: A review. *Environmental Pollution*, *292*, 118319. https://doi.org/10.1016/j.envpol.2021.118319

Ganogpichayagrai, A., & Suksaard, C. (2020). Proximate composition, vitamin and mineral composition, antioxidant capacity, and anticancer activity of *Acanthopanax trifoliatus. Journal of Advanced Pharmaceutical Technology & Research*, *11*(4), 179-183. https://doi.org/10.4103/japtr.JAPTR_61_20

Harwig, J., & Scott, P.M. (1971). Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Applied Microbiology, 21*(6), 1011-1016. https://doi.org/10.1128/am.21.6.1011-1016.1971

Huyen, P.T., Tap, N.V., Son, L.T., Phuong, N.D., Long, C.H., My, D.V., . . ., Thin, N.N. (2006). Nghiên cứu khả năng nhân giống và bảo tồn ngũ gia bì hương và ngũ gia bì gai ở Việt Nam [Research on the possibility of propagating and preserving *Eleutherococcus nodiflorus* and *Eleutherococcus trifoliatus* in Vietnam]. *Tạp chí Dược liệu*, *11*(3), 108-113.

Indrayanto, G., Putra, G.S., & Suhud, F. (2021). Validation of in-vitro bioassay methods: Application in herbal drug research. *Profiles of Drug Substances, Excipients and Related Methodology*, *46*, 273-307. https://doi.org/10.1016/bs.podrm.2020.07.005

Jha, D., Panda, L., Pandian, L., Ramaiah, S., & Anbarasu, A. (2012). Detection and Confirmation of Alkaloids in Leaves of *Justicia adhatoda* and Bioinformatics Approach to Elicit Its Anti-tuberculosis Activity. *Applied Biochemistry and Biotechnology*, *168*. https://doi.org/10.1007/s12010-012-9834-1

Kancheva, V.D., & Kasaikina, O.T. (2013). Bio-antioxidants - a chemical base of their antioxidant activity and beneficial effect on human health. *Current Medicinal Chemistry*, *20*(37), 4784-4805. https://doi.org/10.2174/09298673113209990161

Li, D., Li, Z., Yang, J., Hao, Z., Yang, J., & Lin, C. (2021). Protective Effect of *Acanthopanax trifoliatus* Extract on Rats with Alcoholic Liver Injury. *Science and Technology of Food Industry, 42*(18), 380-386. https://doi.org/10.13386/j.issn1002-0306.2021010238

Li, D.L., Zheng, X., Chen, Y.C., Jiang, S., Zhang, Y., Zhang, W.M., . . ., Zhang, K. (2016). Terpenoid composition and the anticancer activity of *Acanthopanax trifoliatus*. *Archives of Pharmacal Research*, *39*(1), 51-58. https://doi.org/10.1007/s12272-015-0655-y

Li, X., Wu, Q., Xie, Y., Ding, Y., Du, W.W., Sdiri, M., & Yang, B.B. (2015). Ergosterol purified from medicinal mushroom *Amauroderma rude* inhibits cancer growth in vitro and in vivo by up-regulating multiple tumor suppressors. *Oncotarget*, *6*(19), 17832-17846. https://doi.org/10.18632/oncotarget.4026

Lin, Y., Pan, J., Liu, Y., Yang, H., Wu, G., & Pan, Y. (2023). *Acanthopanax trifoliatus* (L.) Merr polysaccharides ameliorates hyperglycemia by regulating hepatic glycogen metabolism in type 2 diabetic mice. *Frontiers in Nutrition*, *10*, 1-13. https://doi.org/10.3389/fnut.2023.1111287

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*, *4*(8), 118-126. https://doi.org/10.4103/0973-7847.70902

Loi, D.T. (2004). *Những cây thuốc và vị thuốc Việt Nam [Vietnamese medicinal plants and herbs]* (D.T. Loi, Ed. Vol. 1). Medical publishing house.

Ly, B., Quan, N., Dao, L., Nguyen, H., Lam, M., & Hoang, C. (2019). Evaluation of Antimicrobial, Antioxidant and Cytotoxic Activities of *Dialium cochinchinensis* Seed Extract. *Indian Journal of Pharmaceutical Sciences*, *81*(5), 975-980. https://doi.org/10.36468/pharmaceutical-sciences.594

MacWillliam, I.C., & Wenn, R.V. (1972). Interpretation of colour tests for polyphenols and melanoidins. *Brewing Industry Research Foundation, Nutfield, Surrey*, 78, 309.

Muhammed, A., Nakalembe, I., Olila, D., Afodun, A., & Mbugua, R. (2021). Anticancer and Apoptotic Potentials of *Gliricidia sepium* Leaf Extract on Breast HCC1395, Prostate DU145 and Colorectal CT26 Cell Lines. *Journal of Cancer and Tumor International*, *11*(4), 89-100. https://doi.org/10.9734/jcti/2021/v11i430163

Mujeeb, F., Bajpai, P., & Pathak, N. (2014). Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of Aegle marmelos. *BioMed Research International*, *2014*, 497606. https://doi.org/10.1155/2014/497606

Muselli, A., Hoi, T.M., Cu, L.D., Moi, L.D., Bessière, J.-M., Bighelli, A., & Casanova, J. (1999). Composition of the essential oil of *Acanthopanax trifoliatus* (L.) Merr. (Araliacaea) from Vietnam. *Flavour and Fragrance Journal, 14*(1), 41-44. https://doi.org/10.1002/(SICI)1099-1026(199901/02)14:1

Nath, M.C., Chakravorty, M.K., & Chowdhury, S.R. (1946). Liebermann-Burchard Reaction for Steroids. *Nature*, *157*(3978), 103-104. https://doi.org/https://doi.org/10.1038/157103b0

Nogueira, F., & Rosário, V.E.d. (2010). Methods for assessment of antimalarial activity in the different phases of the *Plasmodium* life cycle. *Revista Pan-Amazônica de Saúde*, *1*, 109-124.

Peña-Morán, O.A., Villarreal, M.L., Álvarez-Berber, L., Meneses-Acosta, A., & Rodríguez-López, V. (2016). Cytotoxicity, Post-Treatment Recovery, and Selectivity Analysis of Naturally Occurring Podophyllotoxins from *Bursera fagaroides* var. *fagaroides* on Breast Cancer Cell Lines. *Molecules*, *21*(8). https://doi.org/10.3390/molecules21081013

Phuong, N.T., Lee, K.A., Jeong, S.J., Fu, C.X., Choi, J.K., Kim, Y.H., & Kang, J.S. (2006). Capillary electrophoretic method for the determination of diterpenoid isomers in *Acanthopanax* species. *Journal of Pharmaceutical and Biomedical Analysis*, *40*(1), 56-61. https://doi.org/10.1016/j.jpba.2005.05.023

Rieser, M.J., Gu, Z.M., Fang, X.P., Zeng, L., Wood, K.V., & McLaughlin, J.L. (1996). Five novel mono-tetrahydrofuran ring acetogenins from the seeds of Annona muricata. *Journal of Natural Products, 59*(2), 100-108. https://doi.org/10.1021/np960037q

Santos Filipe, M., Isca, V.M.S., Ntungwe, N.E., Princiotto, S., Díaz-Lanza, A.M., & Rijo, P. (2022). Lethality Bioassay using *Artemia salina* L. *Journal of Visualized Experiments : JoVE, 2022 Oct 11*(188). https://doi.org/10.3791/64472

Sithisarn, P., & Jarikasem, S. (2009). Antioxidant activity of *Acanthopanax trifoliatus*. *Medical Principles and Practice*, *18*(5), 393-398. https://doi.org/10.1159/000226294

Thi, T., Quan, N., Thanh Chi, H., & Ly, B. (2023). An Investigation into the Phytochemical Content, Antibacterial Effect, and Antioxidant Capacity of the Ethanol Extract of *Salacca wallichiana* Mart. Peels. *European Journal of Biology*, *82*(2), 179-185. https://doi.org/10.26650/EurJBiol.2023.1316545

Thu, N.T.B., & Tuan, D.T. (2013). Anti-inflammatory property of *Acanthopanax trifoliatus* by suppressing the activity of NF- κB on the macrophagocytes Raw 264.7. *Tạp chí Dược học*, *53*(6), 64-67.

Wang, H.-C., Tseng, Y.-H., Wu, H.-R., Chu, F.-H., Kuo, Y.-H., & Wang, S.-Y. (2014). Anti-proliferation Effect on Human Breast Cancer Cells via Inhibition of pRb Phosphorylation by Taiwanin E Isolated from *Eleutherococcus trifoliatus*. *Natural Product Communications*, *9*(9), 1934578X1400900921. https://doi.org/s10.1177/1934578x1400900921

Wang, H.Q., Li, D.L., Lu, Y.J., Cui, X.X., Zhou, X.F., Lin, W.P., . . ., Zheng, X. (2014). Anticancer activity of *Acanthopanax trifoliatus* (L) Merr extracts is associated with inhibition of NF-kB activity and decreased Erk1/2 and Akt phosphorylation. *Asian Pacific Journal of Cancer Prevention*, *15*(21), 9341-9346. https://doi.org/10.7314/apjcp.2014.15.21.9341

Weerapreeyakul, N., Nonpunya, A., Barusrux, S., Thitimetharoch, T., & Sripanidkulchai, B. (2012). Evaluation of the anticancer potential of six herbs against a hepatoma cell line. *Chinese Medicine*, *7*(1), 15. https://doi.org/10.1186/1749-8546-7-15

Wiart, C. (2007). *Ethnopharmacology of medicinal plants: Asia and the Pacific*. Springer Science & Business Media.

Zhang, X., Bai, Y., Wang, Y., Wang, C., Fu, J., Gao, L., . . ., Deng, Y. (2020). Anticancer Properties of Different Solvent Extracts of *Cucumis melo* L. Seeds and Whole Fruit and Their Metabolite Profiling Using HPLC and GC-MS. *BioMed Research International*, *2020*, 5282949. https://doi.org/10.1155/2020/5282949

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Research Article

Chemical characterization and antioxidant activities of essential oil of *Ficus elastica* Roxb. ex Hornem. leaves

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Abstract: The present study investigates the chemical constituents and antioxidant potential of essential oils extracted from *Ficus elastica*. The essential oils were obtained through steam distillation and were subjected to gas chromatography-mass spectrometry (GC-MS) analysis to determine their constituents representing 100% of the total peak areas. The analysis revealed the presence of 34 compounds. Among these compounds, Caryophyllene was found to be the major constituent, accounting for 50.57% of the total percentage area. Among other identified major compounds include γ-Muurolene (8.19%), camphene (5.69%), Heptacosane (2.61%), and Heneicosane (2.60%). Furthermore, the antioxidant potential of *Ficus elastica* essential oil was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide radical scavenging methods. The results indicated that *F. elastica*'s essential oil exhibited significant radical scavenging activity when compared to the standard antioxidant, Ascorbic acid. For the highest concentration tested (10 µL/mL), the DPPH scavenging inhibition percentage was 45.26% for *F. elastica* and 90.40% for Ascorbic acid. Similarly, the hydrogen peroxide scavenging activity at 10 µL/mL was found to be 74.90% for *F. elastica* and 90.12% for Ascorbic acid. Additionally, the essential oils demonstrated high radical scavenging and chelating activity. The quantitative DPPH and hydrogen peroxide assays indicate the potent antioxidant activity of *F. elastica* essential oil, making it a promising candidate for further biological and chemical analysis. The isolation of therapeutically active compounds from these essential oils can be pursued, considering their potential role in the management and treatment of various diseases.

1. INTRODUCTION

A nexus between humans, ill-health, and folk medicine cannot be traced from recent times because man has been battling with various forms of diseases and illnesses since his origin (Danjuma & Darda'u, 2013). As generations pass and humans suffer from illnesses, the search for remedies to these diseases propels the development of traditional knowledge of medicines, remedies, and norms that are cheaper and have lesser side effects, hence popular among the people (Zain *et al*., 2013). Resources from plants have gained a wide range of applications in various industrial setups for the production of several useful products for the benefit of

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humankind such as perfume, food and dietary supplements, cosmetics, pharmaceutical drugs, etc. (Chika *et al*., 2022). Essential oils (EOs) and bioactive compounds from plants, herbs, fruit waste, and enzymes of fruits or biomaterials are potential crop protection agents. They are largely composed of bioactive secondary metabolites like monoterpenes, esters, sesquiterpenes, phenols, aldehydes, oxides, and ketones that are synthesized both internally and externally by plants (Dassanayake *et al*., 2021). Essential oils, also called volatile odoriferous oils, are aromatic oily liquids extracted from different parts of plants such as leaves, peels, barks, flowers, buds, seeds, etc. (Lubna *et al*., 2020). Among all methods of producing essential oils, the steam distillation method has been widely used, especially for commercial-scale production (Burt, 2004). EOs have been widely used as food flavors because of their antibacterial, antifungal, antioxidant, antiseptic, anti-inflammatory, anti-carcinogenic, and antimutagenic properties (Basavegowda & Baek, 2021). Essential oils found in many different plants, especially aromatic plants, vary in odor and flavor, which are governed by the types and amount of constituents present in oils (Phakawat & Soottawat, 2014). Numerous essential oils with antioxidant properties must be mentioned here since their use as natural antioxidants is a field of real interest, especially in food science and medicine (Tit & Bungau, 2023). Although many antioxidants are available, most antioxidant formulations contain as BHA (Butylated hydroxyanisole) and BHT (Butylated hydroxytoluene), which are synthetic in nature. In recent years, consumers and food manufacturers have opted for products with "all-natural" labels. The area of natural antioxidants developed enormously in the past decade mainly because of the increasing limitations on the use of synthetic antioxidants and enhanced public awareness of health issues (Nanditha & Prabhasankar, 2009). The addition of EOs to edible products, either by direct mixing or in active packaging and edible coatings, may represent a valid alternative to prevent autooxidation and prolong the shelf life of food (Amorati *et al*., 2013; Alparslan, 2018).

Oxidative stress is a problem in human beings since it not only makes our body cells age but also causes diseases such as cancer that are difficult to treat. One of the ways to make the body healthier is to stop the aging process. This can be done using certain chemicals and metals to prevent oxidation or mop up free radicals from the body, known as antioxidants (Musa, 2008). Reactive oxygen species (ROS) are highly reactive molecules that may lead to tissue damage via several different cellular molecular pathways (Ginting *et al*., 2020). Globally, the use of antioxidants as preservatives has been instrumental in improving the quality and extending the shelf life of muscle foods, especially during processing and storage. A low dietary intake of antioxidant, vitamins, and minerals is responsible for the rise in the incidence of cardiovascular diseases and cancer cases (Salehi *et al*., 2018).

Plants are a rich source of phytochemicals with medicinal properties, rendering them useful for the industrial production of pharmaceuticals and nutraceuticals (Hasnain *et al*., 2022). Many supplements, nutricosmetics, and cosmetics are based on botanical ingredients, many of which have a long history of use in traditional or folk medicine (Michalak, 2022). Increasing the antioxidant intake can prevent diseases and lower health problems (Saikat *et al*., 2010). Research is increasingly showing that antioxidant-rich foods and herbs reap health benefits (Saikat *et al*., 2010, Mayo Clinic, 2023).

F. elastica, commonly known as the rubber tree, is an important medicinal plant belonging to the *Moraceae* family. *F. elastica* plants have been widely planted throughout Asia and possess pharmacological properties such as antioxidant, anti-inflammatory, and anticancer. *Ficus* species are reported to be very rich in flavonoids, essential oils, anthocyanins, tannins, and other phenolic constituents (Ginting *et al*., 2020). *F. elastica* (*Moraceae*) is a widely spread evergreen tree up to 30 m tall. The leaves are 7-20 cm long, with smooth edges and blunt pointed tips. The leaves are about a foot long and are thick with a deep green color. The plant is known locally as the "India rubber tree" (Hari *et al*., 2011). Three species (*F. religiosa*, *F. elastica*, *F. benjamina*) display multiple common attributes: high distribution and abundance in local forests, relevant roles in forest ecology, high tolerance and adaptability to stress conditions,

growth in several types of environments (Solis *et al*., 2015). Nigeria's forests are replete with over 45 different species of *Ficus*. Some of them are *Ficus goliath*, *Ficus capensis*, *Ficus ingens, Ficus glomosa, Ficus lecardi*, and *Ficus elastica*. They can be found in the savanna, rainforest, rivers, and streams (Odunbaku *et al*., 2008). Some *Ficus* species are cultivated for their edible fruits (*Ficus sycomorus* Linn.), while others provide shade and function as ornamental plants (Amgad *et al*., 2015).

Ficus elastica is used to cure skin infections, allergies, anemia, neurodegenerative disorders and hepatic problems, and also used as a diuretic agent (Iqbal *et al*., 2018). This research investigation aims to study the chemical composition and antioxidant potential of essential oils extracted from the leaves of *Ficus elastica*. This involves analyzing the constituents of the essential oils using gas chromatography-mass spectrometry (GC-MS) and evaluating their antioxidant activity using DPPH and hydrogen peroxide radical scavenging methods to determine the potential therapeutic benefits of the essential oils and assess their suitability for further biological and chemical analysis for the management and treatment of various diseases.

2. MATERIAL and METHODS

2.1. Sample Collection

Ficus elastica belongs to the family *Moraceae* and was collected from the bush area of Sukur Settlement, Madagali Local Government Area of Adamawa State, Nigeria. Random leaf samples were collected from different trees in the same location into the sack with appropriate labeling and stored in an ice cooler until being transported to the laboratory for extraction and further analysis (Biswas *et al.,* 2013).

2.2. Sample Preparation

The sample of the Plant was properly washed and allowed to dry for 30 minutes in the laboratory. The leaves were then cut into tiny slices to increase the surface area for contact with solvent during extraction process (Ibrahim *et al.,* 2021).

2.3. Extraction of The Essential Oil

1 kg of leaves of *Ficus elastica* sample collected were washed with distilled water and subjected to extraction to avoid loss of some essential oils as a result of the drying process, and using a modified type of steam distillation apparatus (in which the receiver end of the condenser pass-through another vessel containing ice). The time taken for the isolation of the oil is 2½ hours (Runde *et al.,* 2015). The process was repeated for each plant’s batch until a total mass of 2.6 kg was used for extraction. The sample collected was subjected to steam distillation using the Clevenger-like apparatus according to the British Pharmacopoeia (BP) method (British Pharmacopoeia, 2020). The time taken for the isolation of the essential oil is 2½ hours.

2.4. GC-MS Analysis of Essential Oil

Essential oil extracted from the leaves of *Ficus elastica* was analyzed for its chemical constituents by gas chromatography coupled with mass spectrometry (GC-MS). Agilent 19091S-433 Gas Chromatography-Mass Spectrometry System, operating at a pressure of 11.649 psi equipped with a split-splitless injector, was used. Helium was used as a carrier gas at the flow rate of 1 mL/min. The columns used were HP-5MS, capillary column (30 m × 250 µm × 0.25 µm), stationary phase: 5% phenyl methyl silox. The initial temperature was programmed at 60°C for 0.5 min then 10°C/min to 300°C for 3 min followed by a constant temperature at 310°C for 22.5 min. Sample (0.2 µL) was injected into the column programmed at 310°C and the resolution of components was attained. Identification of components was performed by matching their retention indices and mass spectra with those obtained from the NIST library (Iqbal *et al.,* 2018). The spectrum from the GC-MS is shown in Figure 1.

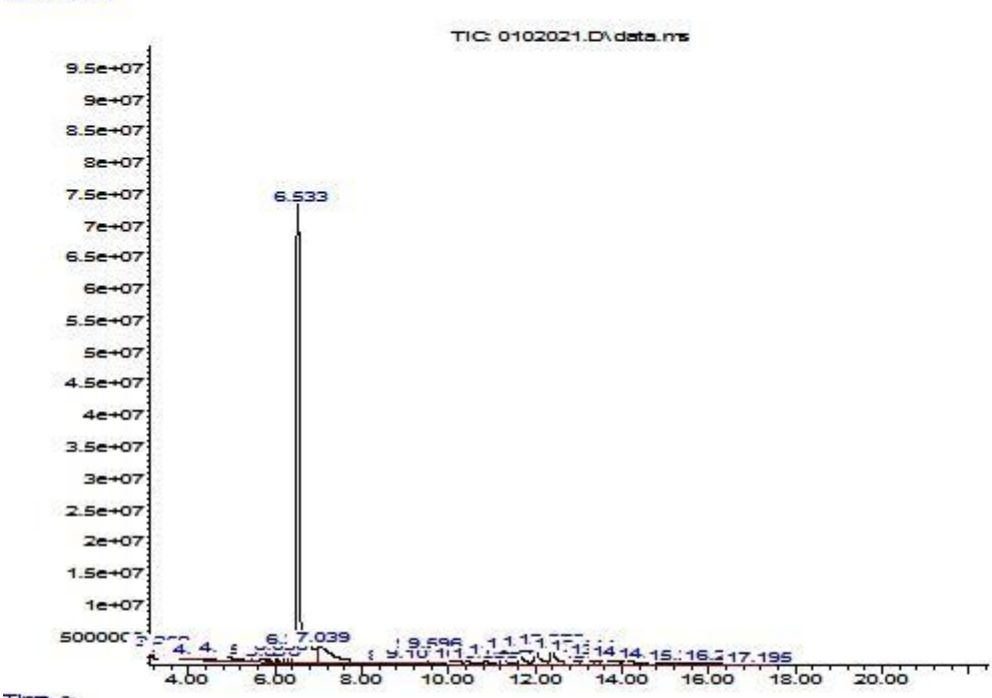

Figure 1. GC-MS Spectrum of essential oil extracted from *Ficus elastica.*

2.5. Determination of Antioxidant Activity of Essential Oils

2.5.1. *Free radical scavenging activity*

DPPH has been widely used for the measurement of the free radical scavenging ability of antioxidants. This method is based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant (Fatiha & Abdelkader, 2019). The DPPH assay was performed using a standard method with minor modification. The hydrogen atom or electron-donating abilities of the compounds were measured from the bleaching of the purple colored methanol solution of 2,2-diphenyl-1-picryl hydrazyl (DPPH). This spectrophotometric assay uses the stable free radical, DPPH as a reagent. One thousand microliters of diverse concentrations (2.5 µL/mL) of the essential oil in ethanol were added to 4 mL of 0.004 % methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm and compared to the standard antioxidants, Ascorbic acid (vitamin C). The DPPH radical scavenging effect was calculated as inhibition of percentage (I %) using the following formula (Burits & Bucar, 2000).

$$I\,\% = \frac{\text{absorbance of Blank} - \text{absorbance of sample}}{\text{absorbance of Blank}}\ x\ 100$$

Where, A is blank is the absorbance of the control reaction (containing all reagents except the test compound) and A (sample) is the absorbance of the test compound. The values of inhibition were calculated for various concentrations of the extract. Tests were conceded out in triplicate.

2.5.2. *Hydrogen peroxide scavenging activity*

The ability of the extract to scavenge hydrogen peroxide was determined according to the method with modification. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (ρH = 7.4). Extracts (2.5-25 µg/mL) in methanol were added to a H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The blank solution contained the phosphate buffer without H_2O_2 (Ebrahimzadeh *et al.,* 2010). The percentage of H_2O_2 scavenging was calculated as:

$$H_2O_2\text{Scavenging effect (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \, x \, 100$$

Where A (control) is the absorbance of the control, and A (sample) is the absorbance in the presence of the sample or standards.

3. RESULTS and DISCUSSION

3.1. Percentage Yield of The Essential Oil from *Ficus elastica*

2.58264 kg of *Ficus elastica* fresh leaves were subjected to steam distillation for the extraction of essential oil components from the plants. The percentage yield was obtained by using the relation

$$\%Yield = \frac{\text{Weight of ail (WO)}}{\text{Weight of plant (WP)}} \, x \, 100$$

The results obtained showed *Ficus elastica* has a percentage yield of 0.09 % as shown in Table 1.

Table 1. Percentage yield of essential oil of *Ficus elastica* (FE) leaves.

Plant	Plant Part Used	Weight of Plant	Weight of Oil	Appearance	% Yield (WO/WP) x 100
FE	Fresh leaves	2.58264 kg	2.38 g	Colorless	0.09

3.2. Chemical Components of Essential Oil of The Plant

The GC-MS analysis of the essential oil of *Ficus elastica* leaves revealed the presence of 34 compounds, which constituted 100% of the total percentage composition. These compounds were listed in order of their retention indexes. The most abundant component was caryophyllene, which is a natural bicyclic sesquiterpene compound accounting for 50.57% of the essential oil. Other components of the essential oil of *Ficus elastica* were γ-Muurolene (8.18%), camphene (5.69%), and heneicosane, 11-decyl- (4.57%) as shown in Table 2. However, this result was not consistent with the outcome of Iqbal *et al*. (2018) study, which revealed that the essential oil of aerial roots of *Ficus elastica* contained compounds such as Benzene, [1-propyldecyl], benzene, benzene, [1-ethylundecyl], propanamide, N,N-dodecyl-3-phenyl, benzene, [1-methyldodecyl], diethyl phthalate, octadecane, 3-ethyl-5-[2-ethylbutyl], and cyclopropane butanoic acid. The difference in composition may be the result of various factors, including the part of the plant used, weather, vegetation, and geographical location (Battaloğlu & Yağız, 2017). Maimuna *et al*. (2016) reported that *Ficus thonningii*'s leaves' essential oil contained 2, 6, 10, 15-tetramethyl-heptadecane (42.42%), 9-methyl-nonadecane (17.62%), eicosane (16.17%), and methylsalicylate (10.58%), which is different from the essential oil composition of *Ficus elastica*. Likewise, Dangarembizi *et al*. (2014) reported that *Ficus thonningii*'s essential oil contained 18.8 % of 6, 10, 14 trimethyl-2-pentadecanone, 14.7% of phytol, 7.6% of acorenone, and 6.3% of β-gurjunene. Similarly, Emmanuel *et al*., (2016) revealed that *Ficus mucoso* contained α-phellandrene (13.0%), p-cymene (11.3%), germacrene D (10.5%), β-caryophyllene (9.7%), 1, 8-cineole (9.5%), and α-copaene (8.7%), which shared some similar composition with *Ficus elastica* in this study.

Adebayo *et al*. (2015) reported that the essential oil composition of *Ficus benghalensis* comprised sesquiterpenes such as α-cadinol (25.1%), germacrene-D-4-ol (14.9%), γ-cadinene (11.8%), and α-muurolene (9.6%), which differ from the result obtained from this research, except for α-muurolene present in *Ficus benghalensis* but was detected in *F. elastica* as γ-muurolene. Sherifat *et al*. (2007) reported that *Treculia africana*'s (Moraceae) essential oil isolated by hydrodistillation and characterized by means of GC-MS contained α-pinene, myrtenal, limonene, camphene, and n-hexanoic acid, which share some common constituents with the findings in this work.

Ogunwande *et al*. (2011) reported the major essential oil composition of *Ficus elastica* Roxb. ex Hornem as 6,10,14-trimethyl-2-pentadecanone (25.9%), geranyl acetone (9.9%), heneicosene (8.4%), 1,8-cineole (8.2%), pentadecanal (6.1%), caryophyllene-oxide (4.2%), (E)-β-ionone (3.9%), and heptadecane (3.3%), which partially agreed with the findings of this research study.

In a separate study, Radulović *et al*. (2016) reported trans-Phytol as the predominant component, constituting 71.2% and 65.4% of the essential oil in fresh and dried leaves, respectively, of *Morus nigra* (Moraceae). Within the same investigation, *Morus alba* (Moraceae) fresh leaves showed the presence of trans-Phytol (61.6%) and Pentacosane (8.2%), while the dried leaves contained nonacosane (12.4%), Hentriacontane (12.4%), Pentacosane (10.9%), and Geranyl acetone (9.8%) as the primary essential oil components. Interestingly, our research findings do not align with these results.

Table 2. GC-MS essential oil analysis of leaves of *Ficus elastica.*

S/N	Constituents	Mol. Formula	RT (Min.)	Area (%)
1	α-Pinene	$C_{10}H_{16}$	3.360	0.77
2	γ-Terpinene	$C_{10}H_{16}$	3.716	0.38
3	α-Phellandrene	$C_{10}H_{16}$	3.973	0.10
4	3-Carene	$C_{10}H_{16}$	4.200	0.86
5	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	$C_{10}H_{16}$	4.821	2.28
6	Cyclohexene, 4-methyl-3-(1-methylethylidene)-	$C_{10}H_{16}$	5.411	1.18
7	2-Carene	$C_{10}H_{16}$	5.525	1.51
8	Methyl m-tolyl carbinol	$C_9H_{12}O$	5.858	0.20
9	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-	$C_{15}H_{24}$	6.062	0.42
10	Copaene	$C_{15}H_{24}$	6.251	1.07
11	1H-Cyclopropa[a]naphthalene, 1a, 2, 3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1aα,7α,7aα,7bα)]-	$C_{15}H_{24}$	6.319	1.19
12	Caryophyllene	$C_{15}H_{24}$	6.531	50.57
13	γ-Muurolene	$C_{15}H_{24}$	7.038	8.19
14	Aromandendrene	$C_{15}H_{24}$	8.529	0.14
15	Z-8-Methyl-9-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	8.673	0.11
16	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	9.037	0.68
17	5β,6β-Epoxy-7α-bromocholestan-3beta-ol	$C_{27}H_{45}BrO_2$	9.097	0.19
18	1,3-Pentadiene, (E)-	C_5H_8	9.354	3.42
19	Camphene	$C_{10}H_{16}$	9.597	5.69
20	Stearic acid hydrazide	$C_{18}H_{38}N_2O$	10.437	0.52
21	Tricosane	$C_{23}H_{48}$	10.861	0.59
22	Tricosane, 2-methyl-	$C_{24}H_{50}$	11.254	1.03
23	Docosane, 5-butyl-	$C_{26}H_{54}$	11.633	1.96
24	Nonadecane	$C_{19}H_{40}$	11.633	1.97
25	Hentriacontane	$C_{31}H_{64}$	12.011	2.41
26	Heptacosane	$C_{27}H_{56}$	12.374	2.61
27	Heneicosane, 11-decyl-	$C_{31}H_{64}$	12.738	2.60
28	Batilol	$C_{21}H_{44}O_3$	13.389	0.57
29	Heptadecane	$C_{17}H_{36}$	13.601	2.21
30	Tetracosane, 3-ethyl-	$C_{26}H_{54}$	14.108	1.46
31	Eicosane	$C_{20}H_{42}$	14.698	1.61
32	Hexadecane, 1-chloro-	$C_{16}H_{33}Cl$	15.394	0.99
33	5-Octadecene, (E)-	$C_{18}H_{36}$	16.219	0.44
34	Octadecane, 1-(ethenyloxy)-	$C_{20}H_{40}O$	17.196	0.08
	Total			100

Contrary to our study, Arsyad *et al*. (2023) outlined the composition of *Ficus elastica* leaf oil, revealing the presence of 1,8-cineole (8.2%), heneicosene (8.4%), geranyl acetone (9.9%), and 6,10,14-trimethyl-2-pentadecanone (25.9%). Additionally, Orhan *et al*. (2016) highlighted the essential oil components of *Maclura pomifera* (Moraceae), noting that Phytol dominated with percentages of 61.5%, 51.4%, and 69.3% for female leaves, male leaves, and fruits, respectively. Battaloğlu & Yağız (2017) further contributed to this knowledge by reporting Dodecanal (9.05%), Eugenol (8.36%), α-humulene (7.84%), and Octadecane (5.28%) as the major components in the essential oil of *Maclura pomifera*.

In a recent study by Cipriano *et al*. (2021), the predominant essential oil compounds in *Euomgenia uniflora* L. genotypes were revealed, indicating two major groups: Sesquiterpene hydrocarbons (26.05%) and Oxygenated sesquiterpenes (40.90%).

3.3. DPPH Radical Scavenging Activity for Essential Oil of *Ficus elastica* and Ascorbic Acid

In Table 3, the essential oil of the plant's leaves demonstrated concentration dependent antioxidant potential and comparable to standard Ascorbic acid, as determined by the DPPH scavenging assay method. At the lowest concentration (2.5 µL/mL), the percentage inhibitions were 41.31% and 52.56% for *Ficus elastica* and Ascorbic acid, respectively. At the highest concentration (10 µL/mL), these values were 45.26% and 90.40%. Figure 2 illustrates the comparison of essential oil activities against the two test reagents. The plant's essential oil exhibited effective scavenging abilities against DPPH in a concentration-dependent manner.

The antioxidant activity of *Ficus elastica* aligns with the findings of Iqbal *et al*. (2018), indicating that the essential oil's antioxidant activity of aerial roots is concentration-dependent. The hydrogen donation removes the odd electron responsible for radical reactivity, as mentioned by Ahmed *et al*. (2017). Yong *et al*. (2019) reported the antioxidant activity (EC_{50} µg/mL) of *Maclura Tricuspidata* Fruit Steam Distilled Essential Oil, which demonstrated superior properties over synthetic antioxidants BHT and BHA in DPPH (17,065.22 ± 146.27), ABTS (1921.81 ± 49.45), and FRAP (10,638.56 ± 223.33) assays.

Eugenia uniflora L., belonging to the same phylum Tracheophyta as *Ficus elastica*, showed positive antioxidant activity (176.66 to 867.57 µM Trolox Equivalent Antioxidant Capacity) in research by Cipriano *et al*. (2021). Similarly, *Salvia officinalis* essential oil, of the same class *Magnoliopsida* as *Ficus elastica*, exhibited antioxidant capacity of 33.61 % and 84.50 % in DPPH and ABTS analyses, respectively (Mot *et al*., 2022). In a study by Ozkan *et al*. (2010) on *Salvia pisidica*, a plant sharing the same phylum and class taxonomic classification with *Ficus elastica*, essential oils from wild and cultivated forms demonstrated higher reducing power activity (EC_{50} 100.99 and 96.87 µg/mL) and β-Carotene/linoleic acid radical scavenging activity (EC_{50} 63.20 and 72.65 µg/mL) than the values of BHT and Ascorbic acid used as standards. Moreover, not much research has been conducted on the antioxidant activity of essential oil of the *Ficus elastica* specie and the *Ficus* family.

Table 3. Result for percentage inhibition of DPPH by the essential oils and ascorbic acid at 517 nm.

Concentration (µL/mL)	*Ficus elastica (%)*	Ascorbic Acid *(%)*
2.5	41.31	52.56
5.0	41.56	65.26
7.5	44.18	79.06
10.0	45.26	90.40

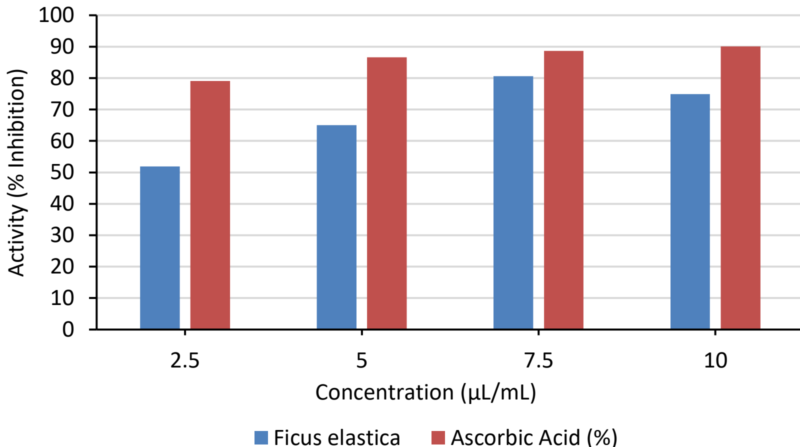

Figure 2. DPPH scavenging activity (%) of *Ficus elastica* essential oils against ascorbic acid.

3.4. Hydrogen Peroxide Radical Scavenging Activity for Essential Oil of *Ficus elastica* and Ascorbic Acid

Table 4 shows that the essential oil of the leaves of these plants demonstrates significant antioxidant potential when compared to the standard Ascorbic acid, as determined by the peroxide scavenging assay method. The percentage inhibitions were 51.92 % and 79.13 % at the lowest concentration (2.5 µL/mL) and 74.90 % and 90.12 % at the highest concentration (10 µL/mL) for *Ficus elastica* and Ascorbic acid, respectively. Figure 3 illustrates the comparison of essential oil activities against the two test reagents.

When comparing the results obtained in this study with the research conducted by Ojah *et al*. (2021) it aligns with their findings on the antioxidant activity of the essential oil of *Calophyllum inophyllum* Linn, belonging to the same class *Magnoliopsida* as *Ficus elastica*. Their study on hydrogen peroxide scavenging assay revealed a concentration-dependent percentage inhibition of the standard used in this study (Ojah *et al*., 2021).

Plumeria alba, belonging to the phylum *Tracheophyta* and class *Magnoliopsida* like *Ficus elastica*, was investigated by Mamattah *et al.* (2023), revealed that the antioxidant activities of the plant's leaves and flower essential oil against hydrogen peroxide exceeded the standard antioxidants used. The IC50 values were 370.5 and 476.0 µg/mL respectively for the flower and leaf parts against the activity of Ascorbic acid (5.30 µg/mL) and Gallic acid (9.08 µg/mL).

In comparing the antioxidant activity of the essential oil of plant leaves against the two test reagents, *Ficus elastica* demonstrated higher hydrogen peroxide radical scavenging efficiency than DPPH radicals, possibly attributed to differences in mechanisms. In both DPPH and hydrogen peroxide assays, the scavenging action may be attributed to the hydrogen-donating ability, as the role of an antioxidant is to eliminate free radicals. No prior research has explored the antioxidant activity of the essential oil of the plant against hydrogen peroxide, serving as a reference for related literature.

Table 4. Result for percentage inhibition of hydrogen peroxide by the essential oils and Ascorbic acid at 230 nm.

Concentration (µL/mL)	*Ficus elastica* (%)	Ascorbic acid (%)
2.5	51.92	79.13
5.0	65.00	86.59
7.5	80.65	88.71
10.0	74.90	90.12

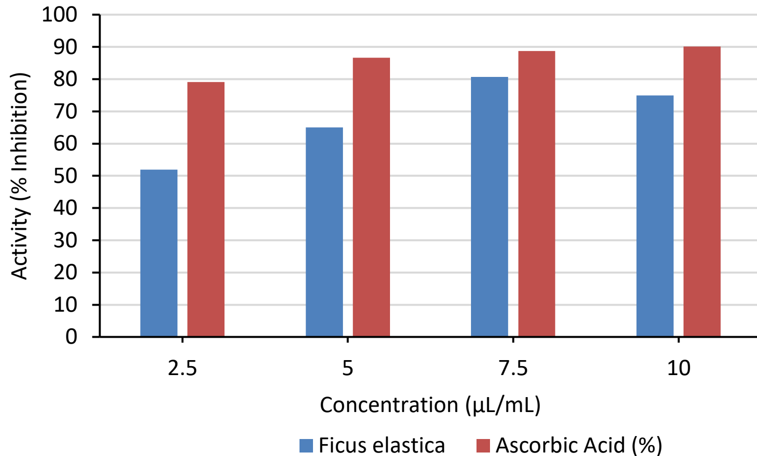

Figure 3. Hydrogen peroxide scavenging activity (%) of *Ficus elastica* essential oils against ascorbic acid.

5. CONCLUSION

Plant essential oils are increasingly utilized in the food industry as natural antioxidant and preservative agents, contributing to enhanced consumer well-being and the prolonged shelf life of consumable food products.

The antioxidant activity of *Ficus elastica* essential oil was examined through DPPH and hydrogen peroxide methods, demonstrating comparable efficacy to standard Ascorbic acid. Quantitative analysis using DPPH and hydrogen peroxide assays revealed potent antioxidant activity within the plant's essential oil, positioning it as a promising candidate for both biological and chemical analyses. Furthermore, the oil could be explored for the isolation of therapeutically active compounds. This robust antioxidant activity suggests potential applications in the management and treatment of various diseases. The findings of this study support the consideration of *Ficus elastica* essential oil as an alternative source of antioxidants.

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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

Author 1 designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. 'Author 1, 2 and 3 managed the analyses of the study. 'Author 1 managed the literature searches. All authors read and approved the final manuscript.

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REFERENCES

Adebayo, A., Aboaba, M.A., Eresanya, S.I., Ajetunmobi, O.A.A., & Ogunwande, A.I. (2015). Constituent of Essential Oil from *Ficus benghalensis* L. *European Journal of Medicinal Plants*, *9*(2), 1-6. https://doi.org/10.9734/EJMP/2015/18803

Ahmed, N.B., Mohamed, G.S., & Adel, G.A. (2017). Antioxidant Activities and Potential Impacts to Reduce Aflatoxins Utilizing Jojoba and Jatropha Oils and Extracts. *International Journal of Pharmacology, 13*(8), 1103-1114. https://doi.org/10.3923/ijp.2017.1103.1114

Alparslan, Y. (2018). Antimicrobial and antioxidant capacity of biodegradable gelatin film forming solutions incorporated with different essential oils. *Journal of Food Measurement and Characterization, 12*(1), 317-322. https://doi.org/10.1007/s11694-017-9643-x

Amgad, I.M.K., Allam, A.E., Nafady, A.M., Ahmad, A.S., & Ramadan, M. (2015). Phytochemical and biological screening of the leaves of *Ficus pandurata Hance* Cultivated in Egypt. *Journal of Pharmacognosy and Phytochemistry*, *4*(1), 50-54.

Amorati, R., Mario, C.F., & Luca, V. (2013). Antioxidant Activity of Essential Oils. *J. Agric. Food Chem*., *61*(46), 10835-10847. https://doi.org/10.1021/jf403496k

Arsyad, A.S., Nurrochmad, A., & Fakhrudin, N. (2023). Phytochemistry, traditional uses, and pharmacological activities of *Ficus elastica* Roxb. ex Hornem: A review. *Journal of Herbmed Pharmacology*, *12*(1), 41-53. https://doi.org/10.34172/jhp.2023.04

Basavegowda, N., & Baek, K.-H. (2021). Synergistic Antioxidant and Antibacterial Advantages of Essential Oils for Food Packaging Applications. *Biomolecules*, *11*(9), 1 18. https://doi.org/10.3390/biom11091267

Battaloğlu, R., & Yağız, F. (2017). Determination of Essential Oils Components of *Maclura pomifera* (Osage Orange) Fruit from Turkey. *International Journal of Secondary Metabolite*, *4*(3), 459-462. https://doi.org/10.21448/ijsm.376898

Biswas, B., Rogers, K., McLaughlin, F., Daniels, D., & Yadav, A. (2013). Antimicrobial Activities of Leaf Extracts of Guava (*Psidium guajava* L.) on Two Gram-Negative and Gram-Positive Bacteria. *International Journal of Microbiology, 2013*, 1-7. https://doi.org/10.1155/2013/746165

British Pharmacopoeia Commission. (2020). British Pharmacopoeia Volume IV. Medicines and Healthcare products Regulatory Agency, London, UK.

Burits, M., & Bucar, F. (2000). Antioxidant activity of *Nigella sativa* essential oil. *Phytotherapy Research*, *14*(5), 323-28. https://doi.org/10.1002/1099-1573(200008)14:5<323::AID-PTR621>3.0.CO;2-Q

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods-a review. *International Journal of Food Microbiology*, *94*(3), 223-253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022

Chika, A., Lawrence, L., Muhammad, F.Y., Kwaji, N., & Polar, T. (2022). An investigation into the chemical composition and in vitro antioxidant activity of ethanolic extracts of *Ficus elastica* and *Mercurialis perennis* leaves. *International Journal of Modern Science and Technology*, *7*(1-2), 6-14.

Cipriano, R.R., Maia, B.H.L.N.S., & Deschamps, C. (2021). Chemical variability of essential oils of *Eugenia uniflora* L. genotypes and their antioxidant activity. *Anais da Academia Brasileira de Ciências*, *93*(1), 1-12. https://doi.org/0.1590/0001-3765202120181299

Dangarembizi, R., Erlwanger, K.H., & Chivandi, E. (2014). Effects of *Ficus thonningii* extracts on the gastrointestinal tract and clinical biochemistry of suckling rats. *Afr. J. Tradit., Complementary Altern. Med., 11*(2), 285-291. https://doi.org/10.4314%2Fajtcam.v11i2.10

Danjuma, M.N., & Darda'u, H. (2013). An Ethno-Survey of Medicinal Trees of Kabobi Village, Northern Katsina, Nigeria. *Academic Research International*, *4*(3), 174-183.

Dassanayake, M.K., Chong, C.H., Khoo, T.-J., Figiel, A., Szumny, A., & Choo, C.M. (2021). Synergistic Field Crop Pest Management Properties of Plant-Derived Essential Oils in Combination with Synthetic Pesticides and Bioactive Molecules: A Review. *Foods*, *10*(9), 5-23. https://doi.org/10.3390/foods10092016

Ebrahimzadeh, M.A., Seyed, M.N., Seyed, F.N., Bahman, E., & Zahra, R. (2010). Antioxidant and Antihaemolytic Activities of the Leaves of *Kefe cumin (Laser trilobum* L) Umbelliferae. *Tropical Journal of Pharmaceutical Research*, *9*(5), 441-449. https://doi.org/10.4314/tjpr.v9i5.61053

Emmanuel, E., Jennifer, M.N., Tameka, M.W., Isiaka, A.O., William, N.S., & Olusegun, E. (2016). Essential oil constituents, anticancer and antimicrobial activity of *Ficus mucoso* and *Casuarina equisetifolia* leaves. *Am. J. Essent. Oil. Nat. Prod.*, *4*(1), 01-06.

Fatiha, M., & Abdelkader, T. (2019). Study of antioxidant activity of *pyrimidinium betaines* by DPPH radical scavenging method. *Journal of Analytical & Pharmaceutical Research*, *8*(2), 33–36. https://doi.org/10.15406/japlr.2019.08.00308

Ginting, C.N., Lister, I.N.E., Girsang, E., Riastawati, D., Hanna, S.W.K., & Wahyu, W. (2020). Antioxidant Activities of *Ficus elastica* Leaves Ethanol Extract and Its Compounds. *Mol Cell Biomed Sci., 4*(1), 27-33. https://doi.org/10.21705/mcbs.v4i1.86

Hari, N.V., Kumar, S.P., & Devi, R.D. (2011). Comparative In-vitro Anthelmintic Activity of the Latex of *Ficus religinosa*, *Ficus elastica* and *Ficus bengalensis. Journal of Phytology*, *3*(3), 26-30.

Hasnain, A., Naqvi, S.A.H., Ayesha, S.I., Khalid, F., Ellahi, M., Iqbal, S., Hassan, M.Z., Abbas, A., Adamski, R., Markowska, D., Baazeem, A., Mustafa, G., Moustafa, M., Hassan, M.E. & Abdelhamid, M.M.A. (2022). Plants in vitro propagation with its applications in food, pharmaceuticals and cosmetic industries current scenario and future approaches. *Frontiers in Plant Science, 13*, 1-21. https://doi.org/10.3389/fpls.2022.1009395.

Ibrahim, M., Ankwai, G.E., Gungshik, J.R. & Taave, P. (2021). comparative extraction of essential oil of *Mentha piperita* (Mint) by steam distillation and enfleurage. *Nigerian Journal of Chemical Research, 26*, 56-63. http//doi.org/10.4314/njcr.v26i2.2

Iqbal, Z., saeed, A., mansha, M, Saeed, K., Afzal, S., Khalil, H.I., Gulzar, Z., & Tara, Z. (2018). Essential Oil from the Aerial Roots of *Ficus elastica* and their Antioxidant Activity. *International Journal of Advanced Research, 6*(1), 137-140. http://dx.doi.org/10.21474/IJAR01/6195

Lubna, A., Khazal, M.A., Nada, K.K.H., & Israa, H.M. (2020). *Essential Oils*. Essential Oils - Bioactive Compounds, New Perspectives and Applications. Edited by de Oliveira, M.S., et al., IntechOpen, 1-11. https://doi.org/10.5772/intechopen.92216

Maimuna, K., Kubmarawa, D., & Aliyu, B.A. (2016). Chemical Constituents of Essential Oil of *Cassia arrereh* Del and *Ficus thoningii* Blume. *International Research Journal of Natural Sciences, 4*(1), 28-30.

Mamattah, K.M.M., Adomako, A.K., Mensah, C.N., & Borquaye, L.S. (2023). Chemical Characterization, Antioxidant, Antimicrobial, and Antibiofilm Activities of Essential Oils of *Plumeria alba* (Forget-Me-Not). *Biochemistry Research International*, *2023*(2), 1-10. https://doi.org/10.1155/2023/1040478

Mayo Clinic. (2007, September 25). *Antioxidants: Preventing Diseases, Naturally*. Science Daily. www.sciencedaily.com/releases/2007/09/070908001613.htm

Michalak, M. (2022). Plant-Derived Antioxidants: Significance in Skin Health and the Ageing Process. *International Journal of Molecular Sciences*, *23*, 1-29. https://doi.org/10.3390/ijms23020585

Mot, M-D., Gavrilas, S., Lupitu, A.I., Moisa, C., Chambre, D., Tit, D.M., Bogdan, M.A., Bodescu, A-M., Copolovici, L., Copolovici, D.M., & Bungau, S.G. (2022). *Salvia officinalis* L. Essential Oil: Characterization, Antioxidant Properties, and the Effects of Aromatherapy in Adult Patients. *Antioxidants, 11*, 1-15. https://doi.org/10.3390/antiox11050808

Musa, A.A. (2008). Antioxidant and antibacterial Activity of *Commiphora kerstingii* Engl. Stem Bark Extract. *Research Journal of Phytochemistry*, *2*(2), 106-111.

Nanditha, B., & Prabhasankar, P. (2009). Antioxidants in Bakery Products: A Review. *Critical Reviews in Food Science and Nutrition*, *49*(1), 1-27. https://doi.org/10.1080/10408390701764104

Odunbaku, O.A., Ilusanya, O.A., & Akasoro, K.S. (2008). Antibacterial activity of ethanolic leaf extract of Fi*cus exasperata* on *Escherichia coli* and *Staphylococcus albus*. *Scientific Research and Essay, 3*(11), 562-564.

Ogunwande, I.A., Flamini, G., Adefuye, A.E., Lawal, N.O., Moradeyo, S., & Avoseh, N.O. (2011). Chemical compositions of *Casuarina equisetifolia L*., *Eucalyptus toreliana* L. and *Ficus elastica* Roxb. ex Hornem cultivated in Nigeria. *South African Journal of Botany*, *77*, 645–649. https://dx.doi.org/10.1016/j.sajb.2011.02.001

Ojah, E.O., Moronkola, D.O., & Osamudiamen, P.M. (2021). Corrigendum: Antioxidant assessment of characterised essential oils from *Calophyllum inophyllum* Linn using 2,2 diphenyl-1- picrylhydrazyl and hydrogen peroxide methods. *Journal of Medicinal Plants for Economic Development*, *5*(1). https://doi.org/10.4102/jomped.v5i1.138

Orhan, I.E., Senol, F.S., Demirci, B., Dvorska, M., Smejkal, K., & Zemlicka, M. (2016). Antioxidant potential of some natural and semi-synthetic flavonoid derivatives and the extracts from *Maclura pomifera* (Rafin.) *Schneider* (Osage orange) and its essential oil composition. *Turkish Journal of Biochemistry*, *41*(6), 403–411. https://doi.org/10.1515/tjb-2016-0125

Ozkan, A., Erdoga, A., Sokmen, M., Tugrulay, S., & Unal, O. (2010). Antitumoral and antioxidant effect of essential oils and in vitro antioxidant properties of essential oils and aqueous extracts from *Salvia pisidica*. *Biologia, 65*(6), 990-996. https://doi.org/10.2478/s11756-010-0108-5

Phakawat, T., & Soottawat, B. (2014). Essential Oils: Extraction, Bioactivities, and Their Uses for Food Preservation. *Journal of Food Science*, *79*(7), 1231-1249. https://doi.org/10.1111/1750-3841.12492

Radulovića, N.S., Miljkovićb, V.M., Mladenovića, M.Z., & Nikolićb, G.S. (2017). Essential Oils of *Morus alba* and *M. nigra* Leaves: Effect of Drying on the Chemical Composition. *Natural Product Communications*, *12*(1), 115-118.

Runde, M., Kubmarawa, D., & Maina, H. (2015). Compositional analysis and *Antimycobacterium* Tuberculosis Activity of Essential Oil of *Hyptis suaveolens* (Lamiaceae). *Research Journal of Chemical Sciences, 5*(7), 40-44.

Saikat, S., Raja, C., Sridhar, C., Reddy, Y.S.R., & Biplab, D. (2010). Free Radicals, Antioxidants, Diseases and Phytomedicines: Current Status and Future Prospect. *International Journal of Pharmaceutical Sciences Review and Research*, *3*(1), 91-100.

Salehi, A., Miquel, M., Jack, L.A., Antoni, S., Natália, M., Pradeep, K.M., & Javad, S.-R. (2018). Antioxidants: Positive or Negative Actors? *Bimolecules*, *8*(124), 1-11. https://doi.org/10.3390/biom8040124

Sherifat, B.A., Olusegun, E., & Oluwole, O. (2007). Constituents of breadfruit tree (*Treculia africana*) leaves, stem and root barks. *Journal of Essential Oil Bearing Plants, 10*(3), 189-193. https://doi.org/10.1080/0972060X.2007.10643541

Solis, M.J.L., dela Cruz, T.E., & Unterseher, M. (2015). The diverse community of leaf inhabiting fungal endophytes from Philippine natural forests reflects phylogenetic patterns of their host plant species *Ficus benjamina*, *Ficus elastica* and *Ficus religiosa. Mycoscience*, 1-11.

Tit, D.M., & Bungau, S.G. (2023). Antioxidant Activity of Essential Oils. *Antioxidants*, *12*(2), 1-4. https://doi.org/10.3390/antiox12020383

Yong, G-R., Gebru, Y.A., Kim, D-W., Kim, D-H., Han, H-A., Kim, Y-H., & Kim, M-K. (2019). Chemical composition and antioxidant activity of steam-distilled essential oil and glycosidically bound volatiles from *Maclura tricuspidata* Fruit. *Foods*, *8*(12), 659. https://doi.org/10.3390/foods8120659

Zain, U., Musa, K.B., Imam, B.B., & Farzana, B. (2013). Proximate and nutrient analysis of selected medicinal plants of Tank and South Waziristan Area of Pakistan. *African Journal of Pharmacy and Pharmacology*, *7*(5)*, 179-184*. https://doi.org/10.5897/AJPP12.766

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Research Article

Molluscicidal activity of *Nigella sativa* seed, *Azadirachta indica* leaf and *Khaya senegalensis* bark ethanolic extracts against *Bulinus truncatus* snails

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Abstract: In order to control schistosomiasis, a strategy involves eliminating the intermediate host responsible for its transmission. Plant-derived molluscicides have been extensively researched as a cost-effective approach to combat this disease, seeking alternative solutions to synthetic molluscicides. The study aimed to analyze the phytoconstituents and assess the molluscicidal impact of ethanolic extracts on adult *Bulinus truncatus* snails. Dried plant materials were ground into powder, and then extracted using ethanol 96%. Preliminary phytochemical screening was carried out using standard procedures. *In vitro* molluscicidal activity was evaluated for the three plants extracts in different concentrations (125, 250, 500 and 1000 ppm). Every 5 adult snails were placed in a plastic cup containing a specific amount of dechlorinated water with plant sample. Control negative was prepared by placing the snails in dechlorinated water only, the numbers of survived and dead snails were recorded after 24 and 48 hours. The Lethal dose and Standard deviation were calculated. The phytochemical screening revealed the presence of different chemical constituents in the three plants samples. The result showed that all the plants extracts possessed molluscicidal activity against *B. truncatus* snails. The *A. indica* leaf extract was the most effective with LD_{50} of 74.32544 ppm. From the findings of this study, Molluscicide of plant origin could be useful against the common snail species in Sudan; therefore, the selected plants can play a big role in community based schistosomiasis control.

1. INTRODUCTION

Schistosomiasis, also known as Bilharzia, is a health condition often linked to poverty that results in prolonged illness. This disease is contracted when individuals come into contact with freshwater contaminated by the larval stages (cercariae) of parasitic blood flukes known as Schistosomes (Thétiot-Laurent *et al*., 2013). The adult worms, too small to be visible to the naked eye, inhabit the veins that drain the urinary tract and intestines. Globally, Schistosomiasis

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impacts nearly 240 million individuals, with over 700 million people residing in areas where the disease is prevalent.

This infection is most prevalent in tropical and sub-tropical regions, particularly among impoverished communities that lack access to clean drinking water and proper sanitation (Karunamoorthi *et al*., 2018). Urogenital schistosomiasis is caused by *Schistosoma haematobium*, while intestinal schistosomiasis is attributed to *S. guineensis*, *S. intercalatum*, *S. mansoni*, *S. japonicum*, and *S. mekongi*. Schistosomiasis significantly impacts global health, leading to severe morbidity in millions of individuals worldwide (Sulieman *et al*., 2017). Present in Sudan since ancient times, schistosomiasis has become widespread, endemic, and a major public health concern in most Sudanese states (Amin & Abubaker, 2017).

During recent years, the rise in immigration and travel from regions with a high risk of schistosomiasis has resulted in an uptick in imported cases of the disease (Mohamed, 2012). The initial instance of the disease in Sudan was documented by Balfour, who discovered that approximately 17% of children attending a primary school in Khartoum were afflicted with urinary schistosomiasis (Hajissa *et al*., 2019). Currently, schistosomiasis is prevalent throughout all states in Sudan. Its distribution and prevalence have been increasing due to the ongoing expansion of water resource development and the growing movement of the population (Ahmed, 2006; Satti et al., 2022; Mamoun et al., 2016). Since ancient times, people have looked to nature for remedies to treat their illnesses. The use of medicinal plants initially started at an instinctual level, as there was limited knowledge about the causes of diseases and which plants could be used as treatments. Everything was based on experiential knowledge (Ndubisi & Ikechukwu Anthony, 2021; Elbasheir *et al*., 2020). It is estimated that over 50% of current drugs have originated from plants in some capacity. In rural areas, plant-based molluscicides provide an environmentally friendly solution for disease control (Ke *et al*., 2019; Mtemeli *et al*., 2021).

Therefore, the use of plant molluscicides for snail control continues to be one of the preferred methods for managing schistosomiasis. The social, economic, and health impacts of schistosomiasis should not be underestimated (Rinaldo *et al*., 2021). The productivity of rural residents is significantly reduced as they suffer from weakness and lethargy caused by the disease. The high cost and environmental consequences associated with current molluscicides have led to the idea of exploring plants for their natural molluscicidal properties (WHO,1994). This approach aims to find a more affordable and sustainable alternative to synthetic products, making molluscicidal agents more accessible to impoverished communities for treating their water sources. Plant-based molluscicides are environmentally friendly and can be utilized by individuals in rural areas to combat diseases (Qian & Zhou, 2021). *Nigella sativa* L.belongs to the family Ranunculaceae. *N. sativa* seeds have medicinal properties that make them effective in treating worms and skin eruptions. The oil derived from these seeds is also used externally as an antiseptic and local anesthetic (Islam *et al*., 2019). The volatile oil of *N. sativa* showed the highest activity level against both miracidia and cercariae (Abo-Zeid & Shohayeb, 2015). *Azadirachta indica* A.Juss. belongs to family Meliaceae. *A. indica* was found to have molluscicidal effect against *B. pfeifferi*. Crude extract from the plant caused the death of the snails at different concentrations (Mwonga *et al*., 2015). *Khaya senegalensis* (Desr.) A. Juss. belongs to family Meliaceae, it has been traditionally employed in ethnomedicine to address a range of health issues, including rheumatoid arthritis, diarrhoea, cough, emetic, emmenagogue, anti-malarial, jaundice, microbial infections, autoimmune inflammatory diseases, and cancer treatment (Adamu *et al*., 2022). The present work reports the phytochemical composition and molluscicidal activity of ethanolic extracts of *N. sativa*, *A. indica* and *K. senegalensis* on *B. truncatus* snails.

2. MATERIAL and METHODS

2.1. Preparation and Extraction of Plant materials

Nigella sativa seeds were obtained from a local market in Khartoum state, *A. indica* leaves were harvested from the University of Medical Sciences and technology yard and *K. senegalensis* bark was collected directly from the field from western Sudan. The three samples were taxonomically identified and authenticated at the herbarium of the Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), Khartoum -Sudan. The three samples were cleaned, dried, and then they were ground into coarse powder. About 100g of each sample were weighed and extracted with 96% ethanol; the samples were filtered and vacuum concentrated by rotatory evaporator.

2.2. Phytochemical Screening

Preliminary phytochemical screening was performed using standard procedures (Evans, 2002), the various extracts were tested for alkaloids, flavonoids, sterols, triterpenes, tannins, saponins, cardiac glycosides, coumarins, anthraquinones, reducing sugars and lignin's.

2.3. Snails Collection and Preparation

A total of 250 adult snails of the species *B. truncatus* were collected from Alfityhab area in East Nile District, Khartoum-Sudan, using a clean bucket with natural water from the snails' habitat. The snails were kept in a wide bucket filled with dechlorinated water in a cool place ready for examination. An experimental *in vitro* study was conducted at the Laboratory of Pharmacognosy Department, Faculty of Pharmacy, University of Medical Sciences and Technology.

2.4. Bioassay

The molluscicidal efficacy test was carried out according to the method described by Ayi *et al* (2019) with some modifications. Every 5 adult snails of the same size were introduced in a plastic cup containing different concentrations of each plant extract (125, 250, 500 and 1000 ppm). Each experiment was repeated in triplicates. Control negative was prepared by placing the snails in 50 ml dechlorinated water only. Snails were exposed to the extracts at room temperature, and then the number of survived and dead snails was recorded after 24 and 48 hours. Death of the snails was determined by lack of movement and discoloration of the shell. After 24 hours and 48 hours, the percentage of dead snails and LD50 values for each extract were calculated , mortality was recorded and all values were expressed as Mean ± SD.

3. RESULTS

3.1. Quantity of Extracts

Different plant samples were extracted with ethanol 96% and percentage yield was calculated. The highest extractive yield was given by *K. senegalensis* bark (16.98%) followed by *N. sativa* (12.3%) while *A. indica* leaves showed the lowest extractive yield (4.19%).

3.2. Qualitative Phytochemical Screening of Plant Extracts

Phytochemical screening of the crude plant extracts of *N. sativa*, *K. senegalensis* and *A. indica* revealed differences in the constituents. *N. sativa* seeds ethanolic extract was found to contain alkaloids, Flavonoids, triterpenes, tannins, Anthraquinones and carbohydrates, while Sterols, saponins, coumarins, cardiac glycosides, reducing sugars and lignin were not detected. On the other hand, the ethanolic extract of the bark of *K. senegalensis* contained alkaloids, triterpenes, tannins, saponins, cardiac glycosides and reducing sugars, but flavonoids, sterols and anthraquinones were not detected. The leaf extract of *A. indica* revealed presence of sterols, tannins and glycosides only while the other compounds were not detected. The results are showed in Table 1.

Table 1. Phytochemical screening of *Nigella sativa*, *Azadirachta indica* and *Khaya senegalensis* extracts.

Test	Sample		
	N.sativa	*K. senegalensis*	*A. indica*
Alkaloids	+ve	+ve	-ve
Flavonoids	+ve	+ve	-ve
Sterols	-ve	-ve	+ve
Triterpenes	+ve	+ve	-ve
Tannins	+ve	+ve	+ve
Saponins	-ve	+ve	-ve
Coumarins	-ve	+ve	-ve
Cardiac Glycosides	-ve	-ve	-ve
Reducing sugars	-ve	+ve	-ve
Anthraquinones	+ve	-ve	-ve
Lignin	-ve	-ve	-ve
Carbohydrates	-ve	+ve	-ve

*Key: (+ve) Detected; (-ve) Not detected

3.3. Molluscicidal Activity of The Crude Extracts

The impact of the tested extracts on *B. truncatus* snails was assessed by tallying the deceased snails. All crude ethanolic extracts displayed a significantly increased in snail mortality at various concentrations. The findings indicated that mortality is dependent on time; furthermore, higher concentrations of the extract led to a significant rise in the mortality rate.

3.3.1. *Nigella sativa*

Molluscicidal activity of *N. sativa* seed extract is presented in Table 2 and Figure 1. The concentrations 1000 ppm and 500 ppm showed a high mortality percentage of 66.67% ± 0.00% in day 1 and 100% in day 2 with an average mortality of 83.33% ± 23.57%.

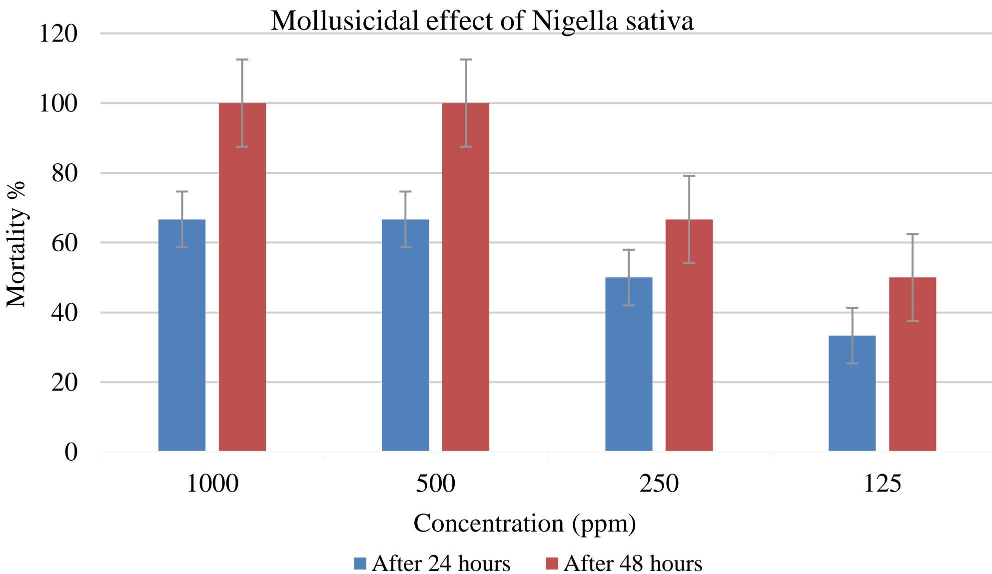

Figure 1. Mortality percentage of *Nigella sativa* seed extract.

Table 2. Molluscicidal effect of *Nigella sativa* seeds extract on *Bulinus truncatus* snails.

Concentration (ppm)	Mortality %		
	After 24 hours	After 48 hours	Mortality average%
1000	66.67±0.00	100±0.00	83.33±23.57
500	66.67±0.00	100±0.00	83.33±23.57
250	50±23.57	66.667±0.00	58.33±11.78
125	33.33±0.00	50±23.57	41.66±11.78
Control -ve (Water)	0 ±0.00	0±0.00	0 ±0.00

3.3.2. *Azadirachta indica*

A. indica leaf extract showed higher toxicity in day 2 with complete death of snails at concentrations 1000 and 500 ppm. The concentration 1000 ppm showed the highest mortality percentage of 91.67 %±11.78%, followed by 500 ppm and 250 ppm with 75%±11.78%, while the lowest concentration (125 ppm) gave the lowest average mortality percentage. The result is shown in Table 3 and Figure 2.

Table 3. Molluscicidal effect of *Azadirachta indica* leaf extract on *Bulinus truncatus* snails.

Concentration (ppm)	Mortality %		
	After 24 hours	After 48 hours	Mortality average%
1000	83.33±23.57	100±0.00	91.67±11.78
500	66.67±0.00	100±0.00	75±11.78
250	66.667±47.14	83.333±23.57	75±11.78
125	50±70.71	66.66±47.14	58.33±11.78
Control -ve (Water)	0 ±0.00	0±000	0 ±0.00

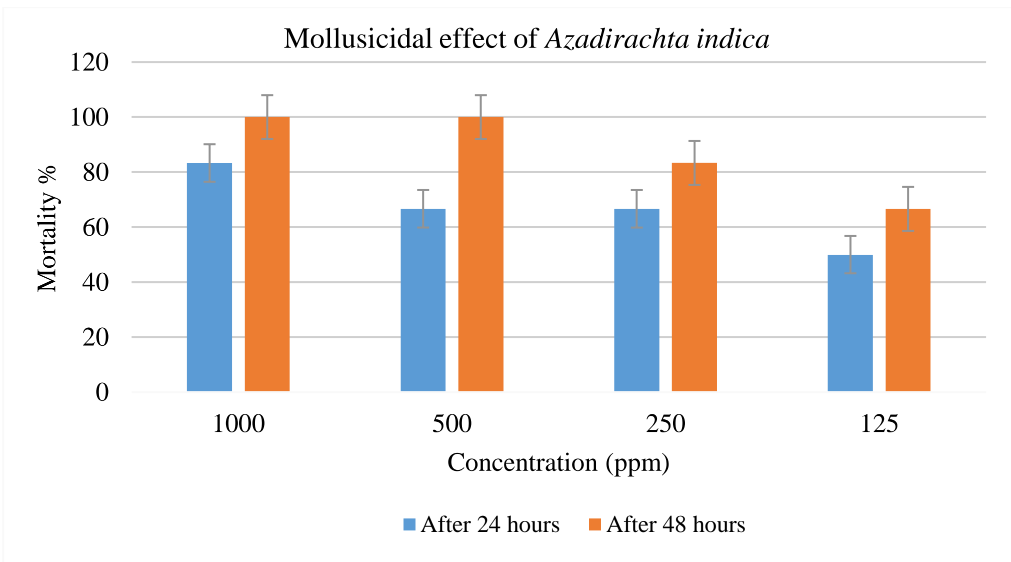

Figure 2. Mortality percentage of *Azadirachta indica* leaf extract.

3.3.3. *Khaya senegalensis*

The *K.senegalensis* achieved the highest mortality level between the three extracts. The results showed in Table 4 and Figure 3. The mortality percentage of the snails was found to be 94.44% ± 7.85% at concentration 1000 ppm, followed by 83.3% ± 7.85 at 500 and 250 ppm respectively.

Table 4. Molluscicidal effect of *Khaya senegalensis* bark extract.

Concentration (ppm)	Mortality %		
	After 24 hours	After 48 hours	Mortality average%
1000	88.89±19.24	100±0.00	94.44±7.85
500	77.78±19.24	88.89±19.24	83.333±7.85
250	77.78±19.24	88.89±19.24	83.33±7.85
125	44.44±19.24	55.56±19.24	50±7.85
Control -ve (Water)	0 ±0.00	0±0.00	0 ±0.00

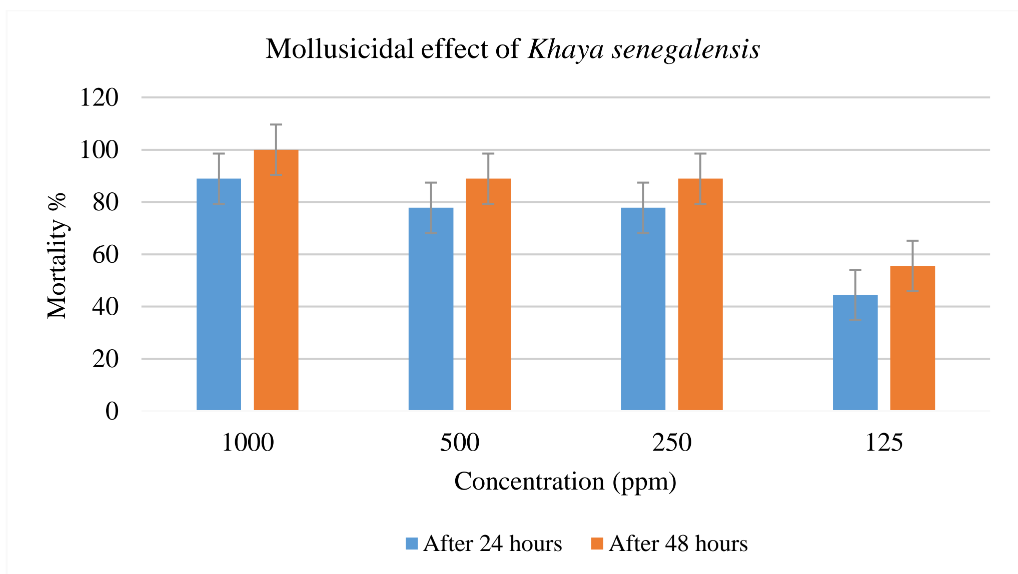

Figure 3. Mortality percentage of Khaya senegalensis bark extract.

3.3.4. *Determination of lethality dose*

LD50 of the extracts was determined to measure the dose that killed 50% of the target organisms within the treatment period. From the present results in this study, the concentrations of plant extracts needed to kill 50%, 90% and 99% of snails were calculated. Among the extracts used on adult snails, *A. indica* leaf extract was the most effective and highly toxic with LD50 of 74.32 ppm. It confirmed that low concentrations were needed to achieve significant death of the snails and that is what makes the extract suitable for use as molluscicide. The results are presented in Table 5.

Table 5. Lethality doses of *Nigella sativa*, *Azadirachta indica* and *Khaya senegalensis* extracts.

Extract	Lethal Doses (ppm)		
	LD_{50}	LD_{90}	LD_{99}
N. sativa	176.77	933.10	1356.60
A. indica	74.32	901.25	1580.10
K. senegalensis	111.36	587.77	854.60

4. DISCUSSION and CONCLUSION

Schistosomiasis continues to pose a significant threat to populations in various parts of the world, particularly in developing countries, where it persists as a major health issue (Verjee, 2019). The search for plants with molluscicidal properties has been intensified, as shown by extensive screening and the overall improved methods and techniques (Barua *et al*., 2021). The

importance of understanding the mode of action of plant molluscicides has been stressed by various investigations (Barua *et al*., 2021), in hope that less toxic, cheaper, available, that could be used in control of snail intermediate host. In this study, the screening of *N. sativa* seed extract, *A. indica* leaf extract and *K. senegalensis* bark extract for molluscicidal potency was carried out. The results showed that all extracts possessed molluscicidal properties against the *B. truncatus* snails. Ethanolic leaf extract of *A. indica* had a stronger molluscicidal activity compared to the other two extracts. This could be attributed to secondary metabolites detected in the phytochemical screening of the extract such as; sterols, tannins and cardiac glycosides. Secondary metabolites in plants are responsible for exhibiting molluscicidal action. Previous researches on plants with molluscicidal properties has focused primarily on two significant secondary metabolites; tannins and saponins. These compounds were considered to be the main toxic substances affecting snails. The exact mechanism of tannins' effect is still not fully understood, but it is believed that they can bind with digestive enzymes and proteins. Eventually, after hydrolysis, tannins can transform into toxic substances within the digestive tract (El-Seedi *et al*., 2022).

On the other hand, saponins cause cell lysis in mollusks, leading to the release of lymph and ultimately resulting in their demise (Quintero Santos *et al*., 2022; Piyasena & Qader, 2022). *K. senegalensis* molluscicidal activity may be due to the presence of flavonoids and terpenoids that were reported in other studies to correlate with the molluscicidal activity (Noorshilawati *et al*., 2020). In addition to the effect of the tannins and saponins that was previously explained. In a previous study, several bioactive phytochemical constituents that have specific physiological effects have been documented, including alkaloids, flavonoids, tannins, terpenoids, saponins, and phenolic compounds (Nigam *et al*., 2020). Most of these phytochemicals were found in the selected plants. The target site of saponins includes muscle, hemolymph, intestine and hepatopancreas (stomach poison) of freshwater snails. Other previous studies have indicated that flavonoids and saponins can hinder the breathing process, potentially by impeding the diffusion of oxygen through the gills of golden apple snails. This obstruction is believed to be caused by the secretion of mucus (Abdullah *et al*., 2017). In another study, alkaloids are highly toxic against vectors of *Schistosomiasis*(Ke *et al*., 2017); cardiac glycosides also proved to have good molluscicidal effect (Dai *et al*., 2011; Kashyap *et al*., 2019).

Utilizing the potential of medicinal plants as natural molluscicides presents a promising alternative to synthetic chemical agents, offering environmentally friendly solutions for controlling mollusk populations and mitigating their adverse effects on human activities. From the observations in the present study, the three plants possessed molluscicidal activity against *B. truncatus* adult snails. Hence, it is the most suitable option for biological applications, providing a potentially simple, readily available, inexpensive, and environmentally safe molluscicidal agent of plant origin for controlling human schistosomiasis in Sudan. Plant-based molluscicides could serve as alternatives to costly synthetic products, enhancing the accessibility of treatment for water collections in impoverished communities. Further research is needed to extract and identify the active components of these plant extracts.

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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

Rawan Osama Abdelaziz: Performed sample collection, Laboratory work and performed analysis. **Ayat Ahmed Alrasheid**: Initiate the idea, participated in analysis, Supervision and wrote the original draft. **Ahmed Saeed Kabbashi**: performed the laboratory work and participated in the analysis. **Gokhan Zengin**: Critical revision and final approval of the article. **Saad Mohammed Hussein Ayoub**: Responsible for conceptualization and investigation of the study, consultation and review the article. All authors have read and approved the final manuscript.

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REFERENCES

Abdullah, N.S., Aziz, N.A., Mailon, R. (2017). Molluscicidal activity of *Entada rheedii* stem bark methanolic extract against paddy pest *Pomacea canaliculata* (Golden Apple Snail). *Malaysian Journal of Analytical Sciences, 21*(1), 46-5. http://dx.doi.org/10.17576/mjas-2017-2101-06

Abo-Zeid, K., Shohayeb, M. (2015). Evaluation of the biocidal activity of alkaloids, saponins and volatile oil extracted from *Nigella sativa* seeds against miracidia and cercariae of *Schistosoma mansoni*. *International Journal of Pharmaceutical Science Invention, 4*(1), 47-54.

Adamu, I., Majiyebo, A.J., Alozieuwa, U.B., Lawal, A.A., Alawode, B.O.O.R.A., & Berinyuy, E.B (2022). Ethnopharmacology, chemical constituents, and biological activities of *Khaya senegalensis* (Desr.) A. Juss a forest plant with multi-purpose applications. *AROC in Natural Products Research, 2*(2), 10-15. https://doi.org/10.53858/arocnpr02021015

Ahmed, A.A. (2006). Schistosomiasis in sugar cane schemes, Sudan. *Sudan J Nat Sci*, *4*, 1-11.

Amin, M., & Abubaker, H. (2017). Control of schistosomiasis in the Gezira irrigation scheme, Sudan. *Journal of Biosocial Science*, *49*(1), 83-98. https://doi.org/10.1017/s0021932016000079

Ayi, Fabrice Chandre, I., Coelho, P.M.Z., El-Harawy, A.K., Elemam, M.A., *et al.* (2019), *Guidelines for laboratory and field testing of molluscicides for control of schistosomiasis*. Rajpal Singh Yadav. World Health Organization, 978 92 4 151540 5. hal-02378870. https://hal.science/hal-02378870

Barua, A., Williams, C.D., & Ross, J.L. (2021). A literature review of biological and bio-rational control strategies for slugs: current research and future prospects. *Insects*, *12*(6), 541. https://doi.org/10.3390/insects12060541

Dai, L., Wang, W., Dong, X., Hu, R., Nan, X. (2011). Molluscicidal activity of cardiac glycosides from *Nerium indicum* against *Pomacea canaliculata* and its implications for the mechanisms of toxicity. *Environmental Toxicology and Pharmacology, 32*(2), 226-32. https://doi.org/10.1016/j.etap.2011.05.007

Elbasheir, M.M., Karti, I.A., & Elamin, E.M. (2020). Evaluation of a rapid diagnostic test for *Schistosoma mansoni* infection based on the detection of circulating cathodic antigen in urine in Central Sudan. *PLoS Neglected Tropical Diseases*, *14*(6), e0008313. https://doi.org/10.1371/journal.pntd.0008313

El-Seedi, H.R., Khalifa, S.A., Mohamed, A.H., Yosri, N., Zhao, C., El-Wakeil, N., & Verpoorte, R. (2022). Plant extracts and compounds for combating schistosomiasis. *Phytochemistry Reviews*, 1-116. https://doi.org/10.1007/s11101-022-09836-x

Esquilla, M.G., Sanchez, C.B., Tenorio, J.C. (2021). Molluscicidal activity of Makabuhay (Tinospora rumphii Boerl) stem ethanolic extract against *Radix* (Lymnaea) spp. Snails. *Journal on New Biological Reports, 10*(2), 64-71.

Evans, W.C. (2009). *Trease and evans Pharmacognosy*. 9th Edition published by Saunders Elsevier. 553.

Hajissa, K., Muhajir, A.E., Eshag, H.A., Alfadel, A., Nahied, E., Dahab, R., ... & Mohamed, Z. (2018). Prevalence of schistosomiasis and associated risk factors among school children in Um-Asher Area, Khartoum, Sudan. *BMC Research Notes*, *11*(1), 1-5. https://doi.org/10.1186/s13104-018-3871-y

Islam, M.T., Khan, M.R., & Mishra, S.K. (2019). An updated literature-based review: Phytochemistry, pharmacology and therapeutic promises of *Nigella sativa* L. *Oriental Pharmacy and Experimental Medicine*, *19*, 115-129. https://doi.org/10.1007/s13596-019-00363-3

Kanu Ikechukwu, K., Ndubisi, E.J.O., & Nwadialor, K.L. (2021). African Religion And Culture Honoring the Past and shaping the future. First Edition. Editors: Kanu, I.A., Nwadialor, K.L., Ndubisi, E.J.O., *Innovative trends in African traditional medicine* (pp. 45-64). Association for the Promotion of African Studies, Maryland.

Karunamoorthi, K., Almalki, M.J., & Ghailan, K.Y. (2018). Schistosomiasis: A neglected tropical disease of poverty: A call for intersectoral mitigation strategies for better health. *Journal of Health Research and Reviews*, *5*(1), 1-12. https://doi.org/10.4103/jhrr.jhrr_92_17

Kashyap, S., Khagta, S., Guleria, K., & Arya, V. (2019). Plants as Molluscicides: A recent update. *International Journal of Botany Studies*, *4*(1), 50-56.

Ke, W., Lin, X., Yu, Z., Sun, Q., Zhang, Q. (2017). Molluscicidal activity and physiological toxicity of *Macleaya cordata* alkaloids components on snail *Oncomelania hupensis*. *Pesticide Biochemistry and Physiology, 143*, 111-115. https://pubmed.ncbi.nlm.nih.gov/29183578/

Ke, W., Tu, C., Cao, D., Lin, X., Sun, Q., & Zhang, Q. (2019). Molluscicidal activity and physiological toxicity of quaternary benzo [c] phenanthridine alkaloids (QBAs) from *Macleaya cordata* fruits on *Oncomelania hupensis*. *PLoS Neglected Tropical Diseases*, *13*(10), e0007740. https://doi.org/10.1371/journal.pntd.0007740

Mutamad, A., Mohamed, K., Noma, M., Hwiada, A., Algali, M., & Mamoun, H. (2016). The Transmission Patterns of Shistosomiasis in Khartoum State, Sudan. *Annals of Clinical Pathology, 4*(6), 1088.

Mohamed, Issam A.W. (February 27, 2012). Water Borne Diseases and Rural Development Study of Bilharzias in Gezira Irrigated Agricultural Scheme, Sudan Available at SSRN: http://dx.doi.org/10.2139/ssrn.2011943

Mtemeli, F. L., Walter, I., Tinago, T., & Shoko, R. (2021). An assessment of the molluscicidal potential of Cucurbita maxima seed extracts on *Biomphalaria pfeifferi* and *Bulinus globosus* snails. *All Life*, *14*(1), 244-255. https://doi.org/10.1080/26895293.2021.1901788

Mwonga, K.B, Waniki, N.E, Dorcas Y.S, Piero, N.M (2015). Molluscicidal effects of aqueous extracts of selected medicinal plants from Makueni County, Kenya. *Pharm Anal Acta., 6*(11), 445-59. https://doi.org/10.4172/2153-2435.1000445

Nigam, M., Mishra, A.P., Adhikari‐Devkota, A., Dirar, AI., Hassan, MM, Adhikari, A., Belwal T., Devkota, H.P. (2020). Fruits of *Terminalia chebula* Retz.: A review on traditional uses, bioactive chemical constituents and pharmacological activities. *Phytotherapy Research*, *34*(10), 2518-2533. https://doi.org/10.1002/ptr.6702

Noorshilawati, A.A., Suraya, A., & Rossiyah, S.S. (2020). Molluscicidal activity *of Ipomoea batatas* leaf extracts against Pomacea canaliculata (Golden apple snail). *Food Research*, *4*(5), 131-137. https://doi.org/10.26656/fr.2017

Piyasena, K.G.N.P., & Qader, M.M. (2022). *Chemistry of Natural Products: Phytochemistry and Pharmacognosy of Medicinal Plants*. Berlin, Boston: De Gruyter, 2022. https://doi.org/10.1515/9783110595949

Qian, Y., & Zhou, X. (2021). *Schistosomiasis Risk and Prevention*. *In Prevention and Control of Infectious Diseases in BRI Countries,* Springer Singapore, 143-156. https://www.infontd.org/resource/schistosomiasis-risk-and-prevention

Quintero Santos, C.D., Castellanos, L., & Becerra-Rozo, W.M. (2022). Possibilities of the alternative management of mollusk pests in agricultural crops. A review. *INGE CUC*, *18*(1), 1-13. http://doi.org/10.17981/ingecuc.18.1.2022.01

Rinaldo, D., Perez-Saez, J., Vounatsou, P., Utzinger, J., & Arcand, J.L. (2021). The economic impact of schistosomiasis. *Infectious Diseases of Poverty*, *10*(1), 1-12. https://doi.org/10.1186/s40249-021-00919-z

Satti, A.B., Mohammed, W.M., Garbi, M.I., & Hamad, M.N.M. (2022). Prevalence of Schistosoma Haematobium Infection in Alsyal Village, River Nile State, Sudan. *SAR Journal of Pathology and Microbiology*, *3*(6), 81-84. https://doi.org/10.36346/sarjpm.2022.v03i06.001

Schenkel, E.P., Gosmann, G., Athayde, M.L. (2007). Saponinas. In: Farmacognosia: da planta ao medicamento. 6ª Porto Alegre: Editora da UFRGS; Florianópolis: Editora da UFSC, 711–740.

Sulieman, Y., Eltayeb, R., Pengsakul, T., Afifi, A., Zakaria, M., & Khairala, M. (2017). Schistosomiasis as a disease and its prevalence in Sudan: An overview. *Journal of Coastal Life Medicine*, *5*(3), 129-133. https://doi.org/10.12980/jclm.5.2017J6-247

Thétiot‐Laurent, S.A.L., Boissier, J., Robert, A., & Meunier, B. (2013). Schistosomiasis chemotherapy. *Angewandte Chemie International Edition*, *52*(31), 7936-7956. https://doi.org/10.1002/anie.201208390

Verjee, M.A. (2019). Schistosomiasis: still a cause of significant morbidity and mortality. *Research and Reports in Tropical Medicine*, *10*, 153-163. https://doi.org/10.2147/RRTM.S204345

World Health Organization. (1994). Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: report of a WHO study group [meeting held in Rome from 22 to 25 June 1992].

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Examination of the chemical composition and anti-diabetic activities of the oils of *Abelmoschus esculentus*, *Peganum harmala*, and *Aquilaria agallocha* cultivated in Muğla

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Abstract: In this study, the oils of *Abelmoschus esculentus*, *Peganum harmala*, and *Aquilaria agallocha* grown in different regions of Muğla, Türkiye were obtained using the cold pressing and maceration techniques. The oils were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) to determine their fatty acid compositions. Thirty-seven fatty acids were detected. Palmitic acid ($C_{16:0}$), linoleic acid ($C_{18:2}$), and oleic acid ($C_{18:1}$ *cis*-9) were the major components in all oils. Additionally, the anti-diabetic activity of the oils was screened against α-amylase and α-glycosidase, which are the related enzymes to diabetes mellitus. Promising results regarding anti-diabetic activity for *Aquilaria agallocha* oils were obtained.

1. INTRODUCTION

The prevalence of type 2 diabetes is rapidly increasing worldwide. There is a consensus among researchers and relevant organizations that lifestyle and dietary factors play a role in the development of the disease. Numerous research data suggest that an increase in saturated and trans fats in the diet increases the risk of coronary heart disease and adversely affects glucose and insulin metabolism, thereby increasing the risk of type 2 diabetes (Baysal, 2011).

Fats are composed of saturated and either monounsaturated or polyunsaturated fatty acids. (Lopez *et al*., 2011). Omega-3 and Omega-6 fatty acids are polyunsaturated fatty acids, both metabolically generating numerous long-chain fatty acids that support various physiological and developmental processes in the body. As they are not synthesized again in humans, they

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must be obtained through diet. Primary dietary sources include plant seeds, nuts, fish oils, and other seafood. Several studies have demonstrated that omega fatty acids are potent molecules that can reduce the risk of cardiovascular diseases and possess anti-cancer, hypolipidemic, anti-inflammatory, and anti-diabetic activities. Omega-6 fatty acids have several health benefits, and when balanced with omega-3 as a dietary supplement, they play a significant role in preventing degenerative diseases and other inflammatory disorders (Gazem *et al*., 2014).

Okra (*Abelmoschus esculentus* L. Moench) is a crucial vegetable plant. Okra is a popular plant recognized to have various health benefits, including anti-diabetic properties (Dubey & Mishra, 2017). Regarding fatty acids, okra seed oil has been reported to be rich in palmitic, oleic, and linoleic acids (Jarret *et al*., 2011). Syrian rue (*Peganum harmala*) has been traditionally and commonly used for medicinal purposes since ancient times. *P. harmala* is reported to have hypoglycemic and cytoprotective effects (Komeili *et al*., 2016). Udi hindi (*Aquilaria agallocha*), an important medicinal plant, is one of the most grown species in the Thymelaeaceae family. It is a rare plant on Earth due to its medicinal properties. The plant has various pharmacological activities such as antinociceptive, antimicrobial, laxative, antioxidant, sedative, anti-hyperglycemic, thrombolytic, anti-diabetic, ulcer protective, anti-cancer, anti-diarrheal, and hepatoprotective activities (Alam *et al*., 2015). Every part of the plant possesses beneficial properties that can serve humanity. Researchers have investigated the anti-diabetic effect of methanol, water, and hexane leaf extracts of *A. agallocha* on streptozocin-induced diabetic rats. The findings suggest that *A. agallocha* leaves have promising potential as an anti-diabetic agent (Pranakhon *et al*., 2011).

The medicinal use of vegetable oils in areas such as health and nutrition is under consideration. Throughout history, it is known that many vegetable oils have been utilized for medical purposes in both written and traditional practices. Diabetes, a recognized chronic disease known to contribute to the onset of various illnesses, lacks a significant therapeutic agent for treating the condition. This situation not only diminishes the quality of life for individuals with diabetes but also exposes them to the concern of developing a new ailment stemming from diabetes. In this context, this study aims to investigate the anti-diabetic activity of the seed oils of *A. esculentus* and *P. harmala* with *A. agallocha* maceration oil, despite the known medicinal properties of these plant species, which are cultivated in the Muğla province and its regions.

2. MATERIAL and METHODS

2.1. Chemicals

All standards used in the study were obtained from Sigma-Aldrich. Other reagents were of analytical-grade purity and were purchased from Merck. Water was purified using a Millipore Milli-Q system involving reverse osmosis, ion exchange, and filtration steps.

2.2. Preparation of Oils

As a part of the research, *A. esculentus* and *P. harmala* seeds, *A. agallocha* cortex were collected from the Muğla-Marmaris and Köyceğiz regions in the year 2023 during their maturation period. The seeds were dried in a plant dryer. Fixed oils of *A. esculentus* (Aeo) and *P. harmala* (Pho) seeds were obtained by passing each seed through an NF 80 cold press machine (Karaerler, Türkiye). The obtained fixed oils were separated from the pulp by filtering. The sterilized oils obtained were stored in the dark at +4°C before use. *A. agallocha* oil (Aao) was obtained by the maceration technique in olive oil. Seeds visuals of *Abelmoschus esculentus* L. Moench (a), *Peganum harmala* (b), and *Aquilaria agallocha* (c) are given Figure 1.

Figure 1. Seeds of *Abelmoschus esculentus* (a), *Peganum harmala* (b), and *Aquilaria agallocha* (c)

2.3. Determination of Fatty Acid Content by GC/MS

An Agilent 7890A GC-5975C MSD was used to determine the fatty acid content of each oil (AOCS 2007). Fatty acid analyses were conducted in accordance with the literature, with modifications to the method described by Kivrak (2020). Sample specimens, each containing 100 mg of oil, were mixed with 10 mL of *n*-hexane and 100 µL of 2 N methanolic potassium hydroxide (Kivrak *et al*., 2020).

The samples were centrifuged at 4000 rpm for 10 min, and the upper layer was transferred to a new tube. Afterward, it was filtered using a single-use 0.20 µm (Macherey-Nagel Chromafil Extra PTFE-20/25 LC) filter disc. Fatty acid methyl esters were analyzed by gas chromatography-mass spectrometry (GC/MS) using an Agilent 7890 GC 5975C inert MSD (Agilent Technologies, Wilmington, DE, USA).

GC/MS analyses were performed using a multi-mode inlet (MMI) (280°C), equipped with a DB-1 capillary column (30 m x 0.25 mm; 0.25 µm), and coupled with an Agilent 5975C using an Agilent 7890 Gas Chromatography. The Mass Spectrometer (MSD) operated in electron impact (EI) mode at 70 eV. The transfer line temperature was set at 280°C, and the carrier gas was helium (2.1 mL/min), while the oven temperature was held at 110°C for 1 min. It was then raised at a rate of 3°C/min to 290°C and maintained for 39 min. The injected volume was 5 µL with a split ratio 40:1. Compound identification was performed by comparing retention times (RT) and mass spectra with the NIST 2008 and Wiley 2008 libraries. Compound percentages were calculated based on peak areas obtained from the MS data.

2.3. Anti-diabetic Activity Assay

2.3.1. *Determining α-amylase inhibitory activity*

α-Amylase inhibitory activity of Aeo, Pho, and Aao was tested spectroscopically with slight modifications to the method presented by Quan *et al*. (2019). In brief, 25 µL of sample solution at different concentrations and 50 µL of α-amylase solution (0.1 U/mL) in a phosphate buffer (prepared with 6 mM NaCl at a pH of 6.9, 20 mM phosphate buffer) were mixed in a 96-well microplate. The mixture was pre-incubated for 10 min at 37°C. After pre-incubation, 50 µL of starch solution (0.05%) was added and incubated for 10 minutes at 37 °C. The reaction was stopped by adding 25 µL of HCl (0.1 M), and then 100 µL of Lugol solution was added for monitoring. A 96-well microplate reader was used to measure the absorbance at 565 nm.

2.3.2. *Determining α-glucosidase inhibitory activity*

α-Glucosidase inhibitory activity of Aeo, Pho, and Aao was determined using a spectroscopic method with slight modifications (Kim *et al*., 2000). In short, 50 µL of phosphate buffer (at pH 6.9; 10 mM), 25 µL of PNPG (*p*-nitrophenyl-α-*D*-glucopyranoside) in a phosphate buffer (at pH 6.9; 10 mM), 10 µL of the sample solution, and 25 µL of α-glucosidase (0.1 U/mL) in a phosphate buffer (at pH 6.0; 10 mM) were mixed in a 96-well microplate. After 20 min of

incubation at 37 °C, 90 µL of sodium carbonate (100 mM) was added to each well to stop the enzymatic reaction. The absorbance was recorded at 400 nm using a 96-well microplate reader.

2.4. Statistical Analysis

The data of all biological activities, however, were given as average of three parallel measurements, respectively. All biological activity assays were carried out at four different concentrations, and the results were presented as IC_{50} values. Data were recorded as mean ± SEM (standard error of the mean) $p<0.01$.

3. RESULTS

3.1. Fatty Acid Compositions

Comparison of fatty acid composition (FAME) results of Aeo, Pho, and Aao are given in Table 1. In the fatty acid content of Aeo, a total of 16 fatty acids have been identified, with linoleic acid (41.03%), palmitic acid (27.96%), and oleic acid (20.58%) as major components. It has been determined to contain 34.44% saturated fatty acids, 24.53% monounsaturated fatty acids, and 41.03% polyunsaturated fatty acids. Omega 6 with linoleic acid (41.03%); omega 7 with palmitoleic acid (0.55%) and *cis*-10 heptadecenoic acid (0.31%); omega 9 with oleic acid (20.58%), elaidic acid (1.35%), *cis*-10-nonadecenoic acid (1.58%), and ricinoleic acid (0.16%) were included.

In the content of Pho, a total of 18 fatty acids have been identified, with linoleic acid (57.15%), oleic acid (25.16%), and palmitic acid (7.58%) as major components. It has been determined that 14.62% are saturated fatty acids, 28.23% are monounsaturated fatty acids, and 57.15% are polyunsaturated fatty acids. Omega 6 with linoleic acid (57.15%), omega 7 with palmitoleic acid (0.19%) and *cis*-10 heptadecenoic acid (0.11%), omega 9 with hypogeic acid (0.09%), oleic acid (25.16%), elaidic acid (2.05%), *cis*-10-nonadecenoic acid (0.16%), and gondoic acid (0.47%) were founded.

In the fatty acid content of Aao, a total of 12 fatty acids have been identified, with oleic acid (64.44%), linoleic acid (14.21%), and palmitic acid (12.18%) being the major components. It has been determined to contain 16.65% saturated fatty acids, 69.14% monounsaturated fatty acids, and 14.21% polyunsaturated fatty acids. Omega 6 with linoleic acid (14.21%), omega 7 with palmitoleic acid (0.64%) and *cis*-10 heptadecenoic acid (0.17%), omega 9 with hypogeic acid (0.12%), oleic acid (64.44%), elaidic acid (2.93%), and gondoic acid (0.43%) were included.

3.2. Anti-diabetic Activity

A comparison of the anti-diabetes activity results of Aeo, Pho, and Aao is given in Table 2. According to the anti-diabetic activity, in the α-amylase inhibition assay, the IC_{50} values of three oils were less than 50 µg/mL. Pho (IC_{50}: 15.36 ± 0.52 µg/mL) and Aao (IC_{50}: 7.38 ± 0.47 µg/mL) were found to be more active than acarbose (IC_{50}: 23.40 ± 0.26 µg/mL), which is the positive standard of the test. In the α-glucosidase inhibition test, all tested oils showed greater activity than acarbose (IC_{50}: 304.36 ± 1.97 µg/mL). Among them, the most active oil, Aao (IC_{50}: 60.40 ± 2.38 µg/mL), was five times more effective than acarbose, while Pho (IC_{50}: 170.29 ± 2.14 µg/mL) exhibited approximately two times better activity than acarbose.

Table 1. Comparison of fatty acid composition (FAME) results of *A. esculentus* (Aeo) and *P. harmala* (Pho) seed oils with *A. agallocha* (Aao) maceration oil.

GC-MS Fatty Acid Compositions							
Peak	RT (min)	Fatty acid (IUPAC Name)	Special Name	Lipid Profile	Aeo (%)	Pho (%)	Aao (%)
1	16.361	Tetradecanoic acid	Myristic acid	C14:0	0.21	0.10	-
2	19.683	Pentadecanoic acid	Pentadecylic acid	C15:0	0.02	0.83	-
3	21.936	7-Hexadecenoic acid	Hypogeic acid	C16:1	-	0.09	0.12
4	22.091	(9*Z*)-9-Hexadecenoic acid	Palmitoleic acid	C16:1	0.55	0.19	0.64
5	23.018	Hexadecanoic acid	Palmitic acid	C16:0	27.96	7.58	12.18
6	25.228	*cis*-10 Heptadecenoic acid	*cis*-10 Heptadecenoic acid	C17:1	0.31	0.11	0.17
7	26.169	Heptadecanoic acid	Margaric acid	C17:0	0.16	0.10	0.10
8	28.216	(9*Z*, 12*Z*)-Octadeca-9,12-dienoic acid	Linoleic acid	C18:2	41.03	57.15	14.21
9	28.472	(9*Z*)-Octadec-9-enoic acid	Oleic acid	C18:1 cis-9	20.58	25.16	64.44
10	28.562	(9*E*)-Octadec-9-enoic acid	Elaidic acid	C18:1 trans-9	1.35	2.05	2.94
11	29.314	Octadecanoic acid	Stearic acid	C18:0	4.89	3.90	3.62
12	30.177	*cis*-10-Nonadecenoic acid	*cis*-10-Nonadecenoic acid	C19:1	1.58	0.16	0.40
13	33.411	12-Hydroxy-9-Octadecenoic acid	Ricinoleic acid	C18:1	0.16	-	-
14	34.202	*cis*-11-Eicosenoic acid	Gondoic acid	C20:1 cis-9	-	0.47	0.43
15	35.138	Eicosanoic acid	Arachidic acid	C20:0	0.70	1.08	0.63
16	37.910	Heneicosanoic acid	Heneicosylic acid	C21:0	-	0.04	-
17	40.576	Docosanoic acid	Behenic acid	C22:0	0.33	0.69	0.12
18	43.148	Tricosanoic acid	Tricosylic acid	C23:0	0.04	0.12	-
19	45.632	Tetracosanoic acid	Lignoceric acid	C24:0	0.13	0.18	-
		Total amount of saturated fatty acids			34.44	14.62	16.65
		Total amount of monosaturated fatty acids			24.53	28.23	69.14
		Total amount of polyunsaturated fatty acids			41.03	57.15	14.21

Table 2. Comparison of anti-diabetic activity results of *A. esculentus* (Aeo), *P. harmala* (Pho), and *A. agallocha* (Aao) oils.

Sample	IC_{50} (µg/mL)	
	α-Amylase inhibitory assay	α-Glucosidase inhibitory assay
Aeo	36.19 ± 1.03	194.33 ± 1.89
Pho	15.36 ± 0.52	170.29 ± 2.14
Aao	7.38 ± 0.47	60.40 ± 2.38
Acarbose*	23.40 ± 0.26	304.36 ± 1.97

* Values expressed herein are mean ± SEM of three parallel measurements ($p<0.05$).
* Reference compounds.

4. DISCUSSION and CONCLUSION

In this study, the oils from the seeds of *A. esculentus* and *P. harmala* grown in different regions of Muğla province were obtained by the cold-pressing technique, while *A. agallocha* oil was prepared by the maceration technique. The oils were analyzed by Gas Chromatography-Mass Spectrometry (GC/MS) to determine their fatty acid compositions. Fatty acids were screened, and it was determined that palmitic acid ($C_{16:0}$), linoleic acid ($C_{18:2}$), and oleic acid ($C_{18:1}$ *cis*-9) were the major components in all three species. Based on the fatty acid content of internal pharmaceuticals containing oils and externally used oils, preparations suitable for different skin types can be formulated. Oils containing saturated fats are suitable for sensitive skins, those with omega 3 are recommended for acne-prone and blemished skins, omega 5-containing oils are utilized for lightening spots and anti-aging, omega 6 containing oils are beneficial for combination skins, and omega 9-containing oils, owing to their ability to penetrate dry skin, can be included in the formulation of cosmetic and aromatherapy preparations. In this context, considering the fatty acid content of the oils analyzed, three oils contain saturated fats. Thus, oils with high omega 6 content, like Aeo and Pho, are suitable for combination skin types, while Aao, containing high levels of omega 9, is considered suitable for preparations intended for dry skin. Aeo, the values of linoleic acid range from 23.6% to 50.65%, while palmitic acid varies between 10.3% and 36.35% (Jarret, *et al*., 2011). In Pho, a total of 17 fatty acid components have been identified, with linoleic acid being the highest at 61.46% (Kaya & Akbas, 2023). It has been found that among the three types of oils, Aao exhibited superior inhibition against both α-amylase and α-glucosidase compared to acarbose. Aleissa *et al.* (2022) reported that the mucilage and extract of *A. esculentus*, traditionally used in the treatment of type 1 diabetes in Traditional Chinese Medicine, demonstrated a significantly advantageous diabetic effect when a single dose of STZ was administered into the peritoneum of Wistar rats compared to okra seed crude extract. The *A. esculentus* extract exhibits anti-diabetic activity, contributing to a general decrease in blood sugar levels. Based on the activity results, they reported that a solid oral formulation could be developed using okra mucilage crude extract and aqueous seed extract obtained from okra as a pharmaceutical excipient (Aleissa *et al*., 2022). The ethanolic extract of *P. harmala* seeds clearly demonstrated a significant reduction in blood sugar levels at doses of 150 and 250 mg/kg in both normal and diabetic rats (Singh *et al*., 2008). They explained that the ethanolic extract of *A. agallocha* flowers effectively inhibited both α-amylase and α-glucosidase enzymes *in vitro* in a dose-dependent manner (Rajagopal *et al*., 2016). The most reactive regions in fatty acids are the carboxyl group and double bonds (Scrimgeour *et al*., 2005). This may be because Pho (85.38% unsaturated fatty acids) and Aao (83.35% unsaturated fatty acids) inhibit both enzymes in their anti-diabetes inhibition activity. Aao holds promising potential as a natural resource for addressing one of today's most significant issues, type-2 diabetes.

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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

Yunus Çetintaş: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft, Methodology, Supervision, and Validation. **Ayşe Çetintaş**: Methodology, Software, Formal Analysis. **Yusuf Sıcak**: Methodology, Software, Formal Analysis. **Mehmet Öztürk**: Methodology, Software, Formal Analysis.

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REFERENCES

Alam, J., Mujahid, M., Rahman, M.A., Akhtar, J., Khalid, M., Jahan, Y., ..., & Iqbal, S.S. (2015). An insight of pharmacognostic study and phytopharmacology of *Aquilaria agallocha*. *Journal of Applied Pharmaceutical Science*, *5*(8),173-181. https://doi.org/10.7324/JAPS.2015.50827

Aleissa, M.S., Mohammed, A.Z., Alneghery, L.M., Hasnain, M.S., Almutairi, B., Ali, D., Alarifi, S., & Alkahtani, S. (2022). Comparative study of the anti-diabetic effect of mucilage and seed extract of *Abelmoschus esculentus* against streptozotocin-induced diabetes in rat model. *Journal of King Saud University-Science*, *34*(8), 102297. https://doi.org/10.1016/j.jksus.2022.102297

AOCS (2007). Free Fatty Acids in Crude and Refined Fats and Oils, Revised 2017, Official Method Ca 5a-40.

Baysal, A. 2011. *Trans yağ asitleri ile insulin direnci ve diyabet etkileşimi [Interaction of trans fatty acids with insulin resistance and diabetes]. Beslenme ve Diyet Dergisi*, *39*(1-2), 5-8. https://doi.org/10.33076/2011.BDD.334

Dubey, P., & Mishra, S. (2017). A review on: Diabetes and okra (*Abelmoschus esculentus*). *Journal of Medicinal Plants Studies, 5*(3), 23-26.

Gazem, R.A.A., & Chandrashekariah, S.A. (2014). Omega fatty acids in health and disease: a review, *Journal of Pharmacy Research*, *8*(8), 1027-44.

Jarret, R.L., Wang, M.L., & Levy, I.J. (2011). Seed oil and fatty acid content in okra (*Abelmoschus esculentus*) and related species. *Journal of Agricultural and Food Chemistry*, *59*(8), 4019-4024. https://doi.org/10.1021/jf104590u

Kaya, E., & Akbas, P. (2023). Determination of the antimicrobial, antioxidant activities and fatty acid composition of *Peganum harmala* Seed. *Brazilian Archives of Biology and Technology*, *66*, e23220237. https://doi.org/10.1590/1678-4324-2023220237

Kim, J.S., Kwon, C.S., & Son, K.H. (2000). Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Bioscience, Biotechnology and Biochemistry, 64*(11),2458-2461. https://doi.org/10.1271/bbb.64.2458

Kıvrak, I., Kivrak, S., & Karababa, E. (2020). Assessment of bioactive compounds and antioxidant activity of Turkey tail medicinal mushroom *Trametes versicolor* (Agaricomycetes). *International Journal of Medicinal Mushrooms*, *22*(6), 559-571. https://doi.org/10.1615/IntJMedMushrooms.2020035027

Komeili, G., Hashemi, M., & Bameri-Niafar, M. (2016). Evaluation of anti-diabetic and antihyperlipidemic effects of *Peganum harmala* seeds in diabetic rats. *Cholesterol,* Article ID 7389864. https://doi.org/10.1155/2016/7389864

Lopez, S., Bermudez, B., Ortega, A., Varela, L.M., Pacheco, Y.M., Villar, J., Abia, R. & Muriana, F.J. (2011). Effects of meals rich in either monounsaturated or saturated fat on lipid concentrations and on insulin secretion and action in subjects with high fasting triglyceride concentrations. *The American Journal of Clinical Nutrition*, *93*, 494-499.

Pranakhon, R., Pannangpetch, P., & Aromdee, C. (2011). Anti-hyperglycemic activity of agarwood leaf extracts in STZ-induced diabetic rats and glucose uptake enhancement activity in rat adipocytes. *Songklanakarin Journal of Science and Technology*, *33*(4), 405-410.

Quan, N.V., Xuan, T.D., Tran, H.D., Thuy, N.T.D., Trang, L.T., Huong, C.T., Andriana, Y., & Tuyen, P.T. (2019). Antioxidant, α-amylase and α-glucosidase inhibitory activities and potential constituents of *Canarium tramdenum* bark. *Molecules*, *24*(3),605. https://doi.org/10.3390/molecules24030605

Rajagopal, P.L., Premaletha, K., & Sreejith, K.R. (2016). Antidiabetic potential of the flowers of *Aquilaria agallocha* Roxb. *World Wide Journal of Multidisciplinary Research and Development*, *2*(4), 22-24.

Scrimgeour, C. (2005). *Bailey's Industrial Oil and Fat Products*, Sixth Edition, Six Volume Set, Chemistry of Fatty Acids, Edited by Fereidoon Shahidi. John Wiley and Sons. 1-5.

Singh, A.B., Chaturvedi, J.P., Narender, T., & Srivastava, A.K. (2008). Preliminary studied on the hypoglycemic effect of *Peganum harmala* seeds ethanol extract on normal and streptozocine induced diabetic rats. *Indian Journal of Clinical Biochemistry*, 23(4), 391-393. https://doi.org/10.1007/s12291-008-0086-3

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Phytochemical screening and *in vitro* biological activity of *Amaranthus viridis* growing in Northern Cyprus

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Abstract: In this study, phenolic and non-phenolic metabolites in different parts of *A. viridis* (from the Turkish Republic of Northern Cyprus) were characterized with reversed-phase high-performance liquid chromatography (RP-HPLC) with diode array detector and high-performance liquid chromatography (HPLC), respectively. In total, approximately twenty-five phenolic compounds including quercetin, chrysin, t-Ferulic acid, and sinapic acid as the most abundant secondary metabolites were identified. On the other hand, four organic acids as non-phenolic compounds quantitatively predominant were identified for the first time in *A. viridis* extracts. The seed and flower extract showed strong ferric-reducing capacity, radical scavenging activity for DPPH˙, phosphomolybdenum assay, metal chelating, and α-amylase inhibition activity by in vitro assays. Our results suggest that *A. viridis* widely used in the human diet in Cyprus is a source of numerous metabolites showing antioxidant and antibacterial potential.

1. INTRODUCTION

Leafy vegetables have been essential in completing both human and animal diets. Vegetables that can be eaten are living examples of the body's nutritious components. They are declared to possess bioactive substances that are physiologically active toward pathogens that lead to diseases (Iqbal *et al.,* 2012; Ahmed *et al.,* 2013; Sunday *et al.,* 2021).

The vegetable *Amaranthus viridis*, often known as "Lindo otu" in Turkish Republic of Northern Cyprus and a member of the Amaranthaceae family, is used for medicinal purposes. It is spread over the world's warmer regions. This plant is thought to have originated in Asia; however, it is occasionally referred to as a global weed that can be found in temperate, tropical, and subtropical regions (Asia, Africa, America, Australia, and Europe) of the world (Khan *et al.,* 2011; Iqbal *et al.,* 2012; Ahmed *et al.,* 2013; Pulipati *et al.,* 2014; Reyad-ul-Ferdous *et al.,* 2015; Sarker & Oba, 2019; Sunday *et al.,* 2021). The plant is a rising, short-lived perennial or

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annual herb that can reach heights of 1 m. It has alternating leaves with petioles that can reach a size of 10 cm. The hairless leaves of *A. viridis* have a dark green top surface and a light green bottom surface. Below 1 mm long, the subglobose fruits are small. The wrinkled, indehiscent, or erratically ruptured sepals are mature. The seeds are 1 mm long, glossy, and dark brown to black. The stem is cylindrical, light green, and has an unpleasant smell. It grows up and is herbaceous. The root's shape is cylindrical, has a strong smell, and is whitish. It has horizontal downward growth and rootlets. Stem and root feature fibrous fracture and a smooth texture (Khan *et al.,* 2011; Pulipati *et al.,* 2014; Reyad-ul-Ferdous *et al.,* 2015; Sunday *et al.,* 2021). It is gathered in the summer season. It is grown on fallow land, roadsides, and farming fields in Bangladesh (Sarker & Oba, 2019), but it grows healthier and more abundant in fertilized and irrigated agricultural land in Northern Cyprus. Due to its flavor, it is a highly very popular green leafy vegetable. Local communities harvest it and use its stem, leaf, flower, and seed for their diet. Broad bean - Lindo, cowpea - Lindo mixture can be boiled or fried with egg and consumed as a meal.

Both the leaves, the seeds, and the stems are very nourishing. Minerals such as phosphorus, iron, calcium, magnesium, zinc, copper, manganese, vitamin A, vitamin C (ascorbic acid), vitamin B_2 (riboflavin), vitamin B_1 (thiamin), fiber content, protein, and amino acids such as cystine, arginine, isoleucine, methionine, tryptophan, histidine, leucine, phenylalanine, tyrosine, lysine, valine, and threonine are among the nutrients found in the leaves. Protein and lipids are present in the seeds. Hence, *A. viridis* attracted a lot of attention due to its excellent nutritional content (Pulipati *et al.,* 2014; Sarker & Oba, 2019).

In addition to these, it is a superb and exceptional source of antioxidant phytochemicals like flavonoids, phenolics, β-carotene, and antioxidant leaf pigments like betalain, β-xanthin, and β-cyanin, as well as a source of other pigments like chlorophylls, anthocyanin, and carotenoids. It was crucial for the food industry because the majority of these chemicals are natural antioxidants that detoxify reactive oxygen species (ROS) in the human body. Significant free radical-scavenging activity is possessed by the betalain, β-xanthin, β-cyanin, amaranthine, and carotenoid pigments. It is very adaptable to many abiotic conditions, including salinity and drought (Iqbal *et al.,* 2012; Ahmed *et al.,* 2013; Torres *et al.,* 2018; Sarker & Oba, 2019; Chen *et al.,* 2022).

Products obtained from *A. viridis* are known to have crucial pharmacological activities. It is believed that the leaves are useful for both cleaning and decreasing tissue swelling. *A. viridis* has antipyretic and analgesic qualities. Therefore, in conventional medicine, it is used to relieve fever and pain. Additionally, this plant has been used to treat ulcers, intestinal bleeding, diarrhea, dysentery, and excessive menstrual flow. The tea prepared from its leaves is used to treat these issues. The plant's seeds have been used to alleviate gastrointestinal issues and decrease labour pain. The plant's leaves are used to treat eczema, burns, wounds, boils, psoriasis, and rashes. The leaves, flowers, and fruits of *A. viridis* perform as a laxative, antileprotic, and anti-inflammatory urinary tract agent, diuretic, antiemetic, antirheumatic, appetite enhancer, respiratory and asthma treatment, ophthalmiatrics, as well as a vermifuge in venereal disorders. They also help to lower cholesterol and excessive blood sugar levels. Moreover, *A. viridis* is utilized for its antidiabetic, antioxidant, hepatoprotective, anti-nociceptive, anti-phytopathogenic, and anthelmintic properties. Other medical uses include those for diaphoretic, galactagogues, gonorrhea, earaches, hemorrhoids, bronchitis, and snake venom antidotes. This plant also has ribosome-inactivating protein, ß-carotene, and antiviral capabilities in addition to antiproliferative and antifungal qualities. These purported antipyretic and analgesic effects, however, are not sufficiently supported by scientific research (Ahmed *et al.,* 2013; Pulipati *et al.,* 2014; Reyad-ul-Ferdous *et al.,* 2015; Olarewaju *et al.,* 2018; Datta *et al.,* 2019; Sarker & Oba 2019; Sunday *et al.,* 2021; Naeem *et al.,* 2022; Zaware *et al.,* 2022).

Since there haven't been any studies on *A. viridis* in Türkiye or Northern Cyprus this study is special and valuable. Hence, the goal of this study is to explore the *A. viridis* methanolic extract's antioxidant, antibacterial, and antidiabetic characteristics.

2. MATERIAL and METHODS

2.1. Gathering and Preparing Plant Material

A. viridis stems, leaves, seeds, and flowers were gathered in July 2022 from Northern Cyprus' Kyrenia region (35°20´35´´N 33°17´09´´E 23 m). The plant was identified via Pl@ntNet. The overall wet weight of the samples was 640 grams. They were then gently split into small pieces and dried for six hours in a 50°C oven. The samples weighed 160 grams in total dry weight. For the analysis, the powdered dried samples were kept in a refrigerator at +4 °C.

2.2. Extraction of Samples

For 1 hour at room temperature, 80% of methanol (1:10 [w/v]) was used to extract the different parts of *A. viridis* on a magnetic stirrer. After that, the Whatman No. 4 paper was used to filter the extract. For biological and chemical investigations, the extracts were stored in a refrigerator at +4 °C.

2.3. Analyses of Antioxidant Activity

2.3.1. *DPPH˙ (1,1-diphenyl-2-picrylhydrazyl) radical scavenging capacity*

Blois (1958), Ucan Turkmen & Mercimek Takci, (2018) stated that the free radical scavenger method relies on the ability of samples to transfer electrons or protons to render the DPPH reagent colorless. 100 µL of the extract was dissolved in 3.9 mL of DPPH˙ reagent (0.025 g/L in methanol) generated in methanol (0.1 mM). To enable the chemical reaction, this mixture was placed in the dark at room temperature for 30 minutes. The mixture's absorbance was measured spectrophotometrically at 517 nm after incubation (Biochrom, Libra S60, B, England). In terms of Trolox equivalent (mg TE/g), DPPH˙ scavenging activity was measured against a methanol blank.

2.3.2. *FRAP (Ferric reducing antioxidant power)*

The ability of antioxidant compounds in the extract to reduce Fe^{3+} to Fe^{2+} was evaluated in this analysis (Oyaizu, 1986). When $FeCl_3$ was added to the reaction mixture, the Prussian blue color that resulted was tested for absorbance. The extract (1 mL) was mixed with 2.5 mL of 1% potassium $K_3Fe(CN)_6$ and 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6). For 20 minutes, this mixture was incubated at 50 °C. 10% trichloroacetic acid (TCA) was added to stop the activity, and the mixture was centrifuged for 10 minutes at 2500 rpm. To 2.5 mL of supernatant, an equal volume of distilled water and 0.5 mL of $FeCl_3$ (0.1%) were added. At 700 nm, the mixture's absorbance was recorded (Biochrom, Libra S60, B, England). The extract's reducing capacity was defined in Trolox equivalents (µg TE/g).

2.3.3. *Performance of metal chelating activity (MCA)*

Dinis *et al.,* (1994) devised a technique to appraise the extract's Fe^{2+} chelating potential. This method's base is the conflict between the extract's metal-binding molecules and ferrozine (a potent iron-chelating agent). Compounds that have a high metal ion binding capacity hinder the red ferrozine/Fe^{2+} complex formation. 100 µL of 2 mM $FeCl_2$, 3.7 mL of distilled water, and 1 mL of extract were combined. 200 µL of 5 mM ferrozine was added to the reaction after it had been incubated at room temperature for 30 minutes. The mixture was then agitated for 10 minutes. The absorbance of the mixture was obtained at 562 nm (Biochrom, Libra S60, B, England). Chelating activity as a percent of inhibition (%) was determined using the formula below.

$$\% \text{ chelating activity} = (1-(A_{sample}/A_{control})) \times 100$$

2.3.4. *Phosphomolybdenum technique*

The phosphomolybdenum technique was used to determine the total antioxidant capacity (TAC) (Zengin *et al.,* 2014). 3 mL of a reactive solution containing 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M sulfuric acid were promptly added to 300 µL of extract. After 90 minutes of incubation at 95 °C, the absorbance was measured at 695 nm (Biochrom, Libra S60, B, England). The total antioxidant capacity was referred to using Trolox equivalents (µg/TE g). At least three times each analysis was performed.

2.4. Total Phenolic (TPC) and Total Flavonoid Content (TFC)

The Folin-Ciocalteu colorimetric procedure was applied to determine the total phenolic content (TPC). Total phenolic content was expressed as gallic acid equivalents (mg GAE)/g (Stanković, 2011). By using the Sharma and Vig method, the total flavonoid content was calculated. Total flavonoid content was expressed as mg of routine equivalent (mg RE)/g (Sharma & Vig, 2013).

2.5. Antibacterial Activity

The extract's antibacterial activity was assessed on Mueller Hinton Agar (MHA) using the approved technique, which adhered to the Kirby-Bauer disc diffusion method, according to Clinical Laboratory Standard Institute (CLSI) recommendations. (CLSI, 2012; Owusu *et al.,* 2021). Turbidity was set to the McFarland standard reference range of 0.5 for the overnight bacterial cultures. 10 µL of each microbial suspension was added to MHA with a pipette and then uniformly applied to the surface with a wooden cotton applicator stick. Placed apart from one another were the sterile blank discs that had been impregnated with 20 µL of the extract. After inoculation, Petri plates were incubated at 37°C for 12 to 24 hours. The inhibition zones surrounding the discs were subsequently measured. Methicillin (M; 5 µg/disc) for *Staphylococcus aureus* ATCC 25923, and Erythromycin (E; 15 µg/disc) for *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* ATCC 25922 were the positive controls. As a negative control, methanol was utilized.

2.6. Assay of α-Amylase Inhibition

The mixture consisted of 1 mL of extract, 1 mL of 20 mM sodium phosphate buffer (pH: 6.9), and 1 mL of 1% w/v starch solution, which was incubated at 37°C for 5 min. The reaction was then started by adding the α-amylase solution (1 mL) to this mixture. After incubation for 30 min., the reaction was terminated with 1 mL of color reagent (96 mM 3,5-dinitrosalicylic acid solution, 2 M NaOH, and 5.31 M sodium potassium tartrate solution). The absorbance of this mixture boiled for 5 minutes, at 540 nm was measured (Biochrom, Libra S60, B, England) (Başyiğit *et al., 2*020). All analyses were repeated three times.

2.7. Phenolic Content

Phenolic components were extracted by adding 100 mL of methanol to approximately 25 g of plant and mixing with extraction solvent (methanol/chloroform/water; 7:2:1) in magnetic stirrers at room temperature. This process was repeated three times. The Whatman filter paper was used to filter the obtained extracts and the filtrates were collected. Then, the solvent was removed at 60 °C in a Buchi, R300 model evaporator. The residues formed at the bottom of the volumetric flask were dissolved in methanol (Zhang *et al.,* 2018).

The reversed-phase high-performance liquid chromatography analysis (Agilent, 1260 Infinity RP-HPLC, USA) was performed to determine the chromatograms of each standard phenolic chemical compound in the extract. The separation of phenolic components was performed on a C18 reverse phase (110 Å, 5 µm, 4.6 x 250 mm, ACE Generix) HPLC column. In the separation process, 10 µl injection volume, mobile phase A (0.1% phosphoric acid-water solution) and B (100% acetonitrile) gradient system, oven temperature 30ºC, and DAD (diode array detector) detector were used (Gupta *et al.,* 2012). The data analysis was done using Agilent Lab Advisor software the phenolic compounds were defined by comparing them with

the standards of each identified compound using retention time. The data obtained are expressed as (mg/kg) dry weight. The analyzes were repeated three times.

2.8. Organic Acid Content

The extraction of organic acids was carried out by the method described by Gallardo-Guerrero *et al.,* (2010). Approximately 1 g of the samples was homogenized in 25 mL deionized water/methanol (7/3, v/v) using a high-speed shredder (IKA, T18 model). The mixture was held in a water bath at 80°C for 30 min. Then, this extract was centrifuged at 10.000 rpm for 10 minutes at 4°C. The supernatant was filtrated through a 0.45 µm filter and transferred into a vial and 20 µL was used for the analysis.

Organic acid analysis was performed by an HPLC (Shimadzu brand, Prominence Modular LC20A model) according to Zong *et al.,* (2016). Separation was done using a Rezex brand, model of ROA-Organic Acid H+ (8%), LC Column 300 x 7.8 mm at 50°C with a UV detector set at 210 nm. The mobile phase was 0.01 N H_2SO_4 at a flow rate of 0.7 mL/min using isocratic flow. The quantification of organic acids was calculated according to the authentic standards and expressed as mg/kg dry weight. All analyses were performed in triplicate.

3. RESULTS

Table 1 presents the results of the methanol extracts of *A. viridis* for total phenolic and flavonoid content, antioxidant activities, and α-amylase inhibition activity. The TPC values of stem, leaf, and seed-flower extracts were calculated as 0.329, 0.665, and 0.516 mg GAE/g, respectively. TFC values of stem, leaf, and seed-flower extracts were calculated as 0.012, 0024, and 0.024 mg RE/g, respectively. DPPH˙ radical scavenging activity of extracts was evaluated by comparing it with the standard antioxidant activity of Trolox equivalent (mg TE/g). DPPH˙ of stem, leaf, and seed-flower extracts was determined as 67, 78, and 82%, respectively (0.43, 0.50, and 0.53 mg TE/g). FRAP results of stem, leaf, and seed-flower extracts were calculated as 23.98, 26.07, and 32.56 µg TE/g, respectively. Metal chelating activity results of stem, leaf, and seed-flower extracts were calculated as 63, 27, and 62%, respectively. Phosphomolybdenum assay results of stem, leaf, and seed-flower extracts were calculated as 191, 210, and 127 µg TE/g, respectively. α-amylase activities of stem and leaf extracts were not detected. On the other hand, α-amylase activities of seed-flower extracts were determined as 2%. The antibacterial efficacy of extracts from *A. viridis* against all tested microorganisms is shown in Table 2.

Table 1. The total phenolic, total flavonoid content, antioxidant, and antidiabetic activities of *A. viridis* methanol extracts.

	Stem extract	Leaf extract	Seed and flower extract
Total phenolic content (mg GAE/g)	0.329±0.002	0.665±0.014	0.516±0.007
Total flavonoid content (mg RE/g)	0.012±0.0003	0.024±0.0007	0.024±0.0003
DPPH (%/mg TE/g)	67±0.022% 0.43±0.022 mg TE/g	78±0.002% 0.50±0.002 mg TE/g	82±0.005% 0.53±0.005 mg TE/g
Ferric reducing capacity (µg TE/g)	23.98±0.066	26.07±2.068	32.56±1.155
Metal (Fe^{2+}) chelating activity (%)	63±0.012	27±0.0005	62±0.006
Phosphomolybdenum (Total antioxidant capacity) (µg TE/g)	191±0.529	210±4.696	127±1.257
α-amylase activity (%)	ND	ND	2±0.025

Values are mean ± Standard deviation (SD) of three replicate analyses. ND: Not detected.

Table 2. Diameter of the inhibition zone (mm) of *A. viridis* extracts.

	A. viridis stem extract	*A. viridis* leaf extract	*A. viridis* seed and flower extract	Positive Control	Negative Control
Staphylococcus aureus ATCC 25923	-	-	-	- (Methicillin)	-
Salmonella typhimurium ATCC 14028	-	-	-	18 (Tetracycline)	-
Escherichia coli ATCC 25922	-	-	-	18 (Tetracycline)	-

(-) represents a no-inhibition zone against microorganisms.

Table 3 displays the identified compounds with their retention times and the amount (mg/kg) of the phenolic content of the methanol extracts of *A. viridis* by reversed-phase high-performance liquid chromatography. In RP-HPLC, twenty-five, twenty-four, and twenty-one compounds were identified for stem, leaf, and seed and flower extracts, respectively. The most abundant compounds in stem extract were quercetin (125.05 mg/kg) and chrysin (112.45 mg/kg). Moreover, the most abundant compounds in leaf extract were catechine hydrate (608.24 mg/kg), t-Ferulic acid (344.8 mg/kg), sinapic acid (233.6 mg/kg), and quercetin (167.2 mg/kg). On the other hand, the most abundant compounds in seed and flower extract were catechine hydrate (674.25 mg/kg), catechine (547.63 mg/kg), t-Ferulic acid (337.7 mg/kg), and sinapic acid (269.13 mg/kg). Apart from these compounds, all compounds found in different amounts are listed in Table 3.

Table 3. The retention time (min) (R.T.) and amount (mg/kg) of the phenolic content of *A. viridis* methanol extracts.

Compound	Stem extract		Leaf extract		Seed and flower extract	
	R.T. (min)	Amount mg/kg	R.T. (min)	Amount mg/kg	R.T. (min)	Amount mg/kg
Chlorogenic acid	6.119	-	6.114	10.64	6.147	13.37
Catechine	6.479	60.75	6.469	54.64	6.668	547.63
Catechine hydrate	6.479	73.6	6.469	608.24	6.668	674.25
Caffeic acid	9.285	9.85	9.657	48.64	9.687	27.37
Vanillic acid	9.843	8.71	9.657	60.08	9.687	33.77
Gentisic acid	10.419	-	10.204	32.24	10.419	-
4-hydroxy benzoic acid	13.776	10.45	13.758	6	13.738	0
Vanillin	16.470	17.95	16.547	21.2	16.453	12.17
p-Coumaric acid	17.727	25.85	17.713	9.92	17.309	-
Rutin	19.409	-	19.075	0	19.483	15.6
Sinapic acid	20.048	58.7	20.055	233.6	20.090	269.13
t-Ferulic acid	20.669	32.6	20.055	344.8	20.090	337.7
Hyperoside	20.669	44.6	20.757	64.8	20.944	-
Isoquercitrin	21.764	14.95	21.796	24.8	21.900	14
Hdroxy cinnamic acid	23.274	17.85	23.686	8.8	23.378	-
Naringin	27.697	9.65	27.716	34.96	27.498	-
o-Coumaric acid	28.462	22.6	28.688	27.44	28.477	20.57
Protocatechuic acid ethyl ester	29.951	7.95	30.165	16.8	30.152	10.51
Rosmarinic acid	30.393	6.2	30.165	8	30.152	5.49
Salicylic acid	31.526	3.55	31.961	27.04	31.960	13.66
Resveratrol	32.436	6.47	32.478	14.4	32.475	2.80
Baicalin	32.802	16.8	32.478	3.52	32.475	6.29

Oleuropein	34.787	-	34.787	-	34.787	-
Quercetin	34.966	125.05	34.965	167.2	34.971	71.2
t-Cinamic acid	35.759	-	35.759	-	35.771	31.6
Naringenin	36.313	12.1	36.312	-	36.413	2.29
Hesperidin	36.850	2.89	36.759	-	36.593	8.40
6-hydroxy flavone	38.113	6.95	37.944	-	37.944	-
Chrysin	39.357	112.45	39.278	30.64	39.502	10.86
Curcumin	39.973	-	39.973	-	39.973	-
Flavones	40.909	2.2	40.909	17.6	40.770	-

Table 4 exhibits the identified compounds with their retention times and the amount (mg/kg) of the organic acid of *A. viridis* methanol extracts by reversed-phase high-performance liquid chromatography. In HPLC, 3 compounds were identified for all extracts: Succinic acid (28112.65 mg/kg), malic acid (12513.75 mg/kg), and shikimic acid (269. mg/kg) were present in stem extract. Additionally, leaf and seed-flower extracts contained shikimic acid (837.2 and 291.58 mg/kg), succinic acid (72992.48 and 30212.26 mg/kg), and formic acid (3943.84 and 2727.23 mg/kg).

Table 4. The retention time (min) (R.T.) and amount (mg/kg) of organic acid of *A. viridis* methanol extracts.

Compound	Stem extract		Leaf extract		Seed and flower extract	
	R.T. (min)	Amount mg/kg	R.T. (min)	Amount mg/kg	R.T. (min)	Amount mg/kg
Malic acid	10.622	12513.75	10.541	-	10.541	-
Shikimic acid	12.835	269.3	12.860	837.2	12.864	291.58
Succinic acid	12.835	28112.65	12.860	72992.48	12.864	30212.26
Formic acid	14.501	-	14.913	3943.84	14.914	2727.23

4. DISCUSSION and CONCLUSION

The secondary metabolites have anti-inflammatory, antioxidant, immune system stimulation, detoxification enzyme modulation, steroid metabolism, antiviral, anticancer, and antibacterial activities as natural compounds in plants. Table 1 shows the antioxidant activity and total phytochemical contents of methanol extracts of different parts of *A. viridis*. Leaf extract showed a high level of total phenolics (0.665±0.014 mg GAE/g) and had pronounced levels of radical scavenging (78±0.002%), and ferric reducing (26.07±2.068 µg TE/g) antioxidant activities. Despite the highest DPPH scavenging (82±0.005%) and ferric reducing (32.56±1.155 µg TE/g) capacities, seed and flower extracts had a lower total phenolic content (0.516±0.007 mg GAE/g) than that of leaf extract. The stem, seed, and flower extracts showed good metal-binding potential in the presence of ferrozine (Table 2), % values were 63±0.012 and 62±0.006, respectively. These results indicated that the methanolic extracts belonging to different parts of *A. viridis* contained a strong antioxidant capacity, and their efficacy was comparable with the literature studies.

Some previous studies reported that the high phenolic constituents in *A. viridis* contribute to antioxidant activity (Datta *et al.,* 2019; Sarker & Oba, 2019; *Sunday et al.,* 2021) and this was the case in this study. The superior levels of antioxidant activities of the leaf, seed, and flower extracts with high phenolic content partly confirmed this hypothesis. Pulipati *et al.,* (2014) found that the extracts prepared from the seeds of *A. viridis* had the highest DPPH antioxidant activity, which is consistent with our findings. Similarly, Abdel-alim *et al.,* (2023) declared the high DPPH (123±8.9 mg VCE/g DW) and FRAP (1.5±0.04 mM FE^2+ equivalent/g DW) antioxidant values of *A. viridis* ethanolic extract. Our results were similar with the observation of Popoola, (2022) and Sarker & Oba, (2020) that *A. viridis* seed extracts included radical scavenging agents reacting directly to DPPH. Kumari *et al.,* (2018) also indicated similar

antioxidant results for *A. viridis* extracts, showing that the high DPPH scavenging potential ranged from 53.31±1.08% to 65.2±1.41%. Datta *et al.,* (2019) reported that the DPPH (13.126±0.263%), FRAP (1.553±0.004 TE, mg/g DE) and MCA (22.359±0.491%) capacities of *A. viridis* extracts were quite lower than those of our antioxidant results.

The antioxidant activity of plant foods is now widely acknowledged to be primarily sourced from phenolics. Thus, it is crucial to ascertain the extract's phenolic content. The TP and TF contents of methanolic extracts for *A. viridis* different parts are of the order: for stem 0.329±0.002 mg GAE/g and 0.012±0.0003 mg RE/g, for leaf 0.665±0.014 mg GAE/g and 0.024±0.0007 mg RE/g, and for seed and flower 0.516±0.007 mg GAE/g and 0.024±0.0003 mg RE/g. These values were higher than the reported levels of green Amaranth leaves (ranging from 11.24±00.5 to 20.13±0.04 GAE μg/g) (Sarker *et al.,* 2020). In another study, the total phenolic contents of the *A. viridis* extracts, especially the leaf extract correlated to its high antioxidant activity with 57.25 mg GAE/g (Swarnakumari *et al.,* 2021). Similarly, Popoola, (2022) noted that the amount of total phenolic and flavonoid compounds found in the raw extract from the seed parts of *A. viridis* were 107.9±1.04 mg TA/g and 24.1±0.89 mg RE/g, respectively. The other stated total phenolic contents (TPC) for the methanolic extracts of the leaves *A. viridis* ranged from 40.26±0.27, 43.24±0.32, and 46.72±0.22 GAE μg/g (Sarker & Oba, 2019). The presence, absence, and formation of available phenolic constituents in *A. viridis* vary depending on environmental and genetic factors.

One Gram-positive and two Gram-negative bacterial strains were tested for the antibacterial activity of the *A. viridis* methanol extracts. The antibacterial activity of the extract could not be observed against the tested microorganisms. However, literature studies pointed out that the variety of microorganisms in which *A. viridis* extracts show antimicrobial activity was quite much (Akbar *et al.,* 2018; Rose *et al.,* 2021; Zahir *et al.,* 2021).

A promising therapeutic approach for the management and treatment of chronic health disorders including diabetes and obesity is the inhibition of digestive enzymes like α-amylase. Therefore, the α-amylase activity of extracts was examined. *A. viridis* seed-flower methanol extracts exhibited α-amylase inhibitory activity of 2±0.025%. Oluwagunwa *et al.,* (2021) reported a strong α-amylase inhibition activity (68.45%) in the aqueous leaf extract of *A. viridis* in contrast with our data. Similarly, Mareshvaran *et al.,* (2020) noted the high inhibitory capacity (73.85%) of the α-amylase enzyme of *A. viridis* dichloromethane extract. The findings of earlier investigations demonstrated that *A. viridis* extracts exhibit α-amylase inhibitory activity.

Plants include a large number of phenolic compounds and flavonoids, which have biological properties like antidiabetic (e.g., chlorogenic acid, caffeic acid, and rutin), antimicrobial (e.g., ferulic acid, and sinapic acid), anti-inflammatory (e.g., vanillic acid, ferulic acid, and quercetin), antioxidant (e.g., sinapic acid, p-coumaric acid, and quercetin), antianxiety (e.g., sinapic acid), antihistamine (quercetin) and anticarcinogenic (e.g., p-coumaric acid, chlorogenic acid, ferulic acid, sinapic acid, quercetin, and rutin) (Sadia *et al.,* 2016; Datta *et al.,* 2019;). The commonly identified components of *A. viridis* are flavonoids, cyanogenic glycosides, saponins, tannin, and phlobatannins (Iqbal *et al.,* 2012). Table 3 presents the qualitative results of phytochemicals in different parts of *A. viridis.*

The dominant phenolic compounds of the *A. viridis* extract were catechine, catechine hydrate, and sinapic acid, followed by quercetin. Sarker and Oba (2020), similar to our phenolic content varying amounts depending on the plant parts, identified salicylic acid, gentisic acid, vanillic acid, chlorogenic acid, ferulic acid, t-cinnamic acid, quercetin, p-coumaric acid, caffeic acid, rutin, sinapic acid, naringenin, and catechin by using HPLC. A study conducted by Abdel-alim *et al.,* (2023) expressed the presence of rutin, quercetin, and naringin as major phenolics in the stem and leaves of *A. viridis.* Kumari *et al.,* (2018) and Zaware *et al.,* (2022) reported identifying chlorogenic acid and ferulic acid in *A. viridis* extract.

The phytochemical results from the current study express that the methanolic extracts of stem, leaf, and seed-flower of *A. viridis* included pharmacologically active compounds having varied biological potential. Especially, the most popular among the biologically active compounds in the chemical composition of plants are organic acids. In this study, the efficiency of this method for the extraction of organic acids from *A. viridis* was evaluated for the first time. The potential bioactive organic acid profile of *A. viridis* was determined in scientific reports using different extraction methodologies (Javed *et al.,* 2018). Javed *et al.,* (2018) reported the different organic acid components (citric, malic, fumaric, oxalic, and glutamic acids) of *A. viridis* root exudates in contrast with our data.

The current study reported the phenolic and non-phenolic profiles, antioxidant activity, antibacterial activity, and antidiabetic properties of extracts obtained from different parts of *A. viridis* (Cyprus). The metabolic profile of the extracts obtained from different parts of *A. viridis* was determined by RP-HPLC and nearly thirty-one phenolic compounds were characterized. Contents varying depending on plant parts, two compounds, catechin, and sinapic acid were at the highest level in the phenolic metabolites. Organic acids, non-phenolic compounds, were quantitatively predominant, opposite phenolics. This indicates that the ripening of plant parts is a complex mechanism affecting the quantitative profile of phytochemicals. The phenolic and non-phenolic metabolites identified in *A. viridis* extracts were the source of strong biological activity, in particular phosphomolybdenum activity, metal chelating activity, free radical scavenging capacity, ferric reducing antioxidant power, antibacterial activity, and anti-diabetic impact performed *in vitro* models. Our results highlighted the potential of *A. viridis* as a source of antioxidant phytochemical contents that need more scientific information to explain the molecular mechanisms in biological activities through *in vivo* 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

Melis Sümengen Özdenefe: Concept, Design, Supervision, Resources, Materials, Data collection and/or Processing, Analysis and/or Interpretation, Literature Research, Writing, and Critical Reviews. **Fikret Büyükkaya Kayış**: Concept, Design, Supervision, Analysis and/or Interpretation and Critical Reviews. **Ümit Haydar Erol:** Materials, Data collection and/or Processing, and Analysis and/or Interpretation. **Hatice Aysun Mercimek Takcı:** Concept, Design, Supervision, Resources, Materials, Data collection and/or Processing, Analysis and/or Interpretation, Literature Research, Writing, and Critical Reviews.

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REFERENCES

Abdel-alim, M.E., Serag, M.S., Moussa, H.R., Elgendy, M.A., Mohesien, M.T., & Salim, N.S. (2023). Phytochemical screening and antioxidant potential of *Lotus corniculatus* and *Amaranthus viridis*. *Egyptian Journal of Botany*, *63*(2), 1-17. http://dx.doi.org/10.21608/ejbo.2023.158720.2118

Ahmed, S.A., Hanif, S., & Iftkhar, T. (2013). Phytochemical profiling with antioxidant and antimicrobial screening of *Amaranthus viridis* L. leaf and seed extracts. *Open Journal of Medical Microbiology, 3*, 164-171. http://dx.doi.org/10.4236/ojmm.2013.33025

Akbar, M., Sherazi, I.N., Iqbal, M.S., Khalil, T., & Waqas, H.M. (2018). Antibacterial and antioxidant activities of Slender Amaranth Weed. *Planta Daninha*, *38, e020192974*, 1-8. http://dx.doi.org/10.1590/S0100-83582020380100006

Başyiğit, B., Sağlam, H., Köroğlu, K., & Karaaslan, M. (2020). Compositional analysis, biological activity, and food protecting ability of ethanolic extract of *Quercus infectoria* gall. *Journal of Food Processing and Preservation, 44*, e14692. https://doi.org/10.1111/jfpp.14692

Blois, M.S. (1958). Antioxidant determinations by the use of stable free radical. *Nature*, 1199–1200.

Chen, C.C., Huang, M.Y., Lin, K.H., & Hsueh, M.T. (2022). The effects of nitrogen application on the growth, photosynthesis, and antioxidant activity of *Amaranthus viridis*. *Photosynthetica*, *60*(3), 420-429. http://dx.doi.org/10.32615/ps.2022.034

CLSI, (2012). *Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard* (7th ed.). CLSI document M02-A11, Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

Datta, S., Sinha, B.K., Bhattacharjee, S., & Seal, T. (2019). Nutritional composition, mineral content, antioxidant activity and quantitative estimation of water soluble vitamins and phenolics by RP-HPLC in some lesser used wild edible plants. *Heliyon*, *5*, e01431, 1-37. https://doi.org/10.1016/j.heliyon.2019.e01431

Dinis, T.C., Madeira, V.M., & Almeida, L.M. (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and assay peroxyl radical scavengers. *Archives of Biochemistry and Biophysics, 315*(1), 161–169. https://doi.org/10.1006/abbi.1994.1485

Gallardo-Guerrero, L., Perez-Galvez, A., Aranda, E., Minquez-Mosquera, M.I., & Hornero-Mendez, D. (2010). Physicochemical and microbiological characterization of the dehydration processing of red pepper fruits for paprika production. *LWT - Food Science and Technology*, *43*, 1359-1367. http://dx.doi.org/10.1016/j.lwt.2010.04.015

Gupta, M., Sasmal, S., Majumdar, S. & Mukherjee, A. (2012). HPLC profiles of standard phenolic compounds present in medicinal plants. *International Journal of Pharmacognosy and Phytochemical Research*, *4*(3), 162-167.

Iqbal, M.J., Hanif, S., Mahmood, Z., Anwar, F., & Jamil, A. (2012). Antioxidant and antimicrobial activities of Chowlai (*Amaranthus viridis* L.) leaf and seed extracts. *Journal of Medicinal Plants Research, 6*(27), 4450-4455. https://doi.org/10.5897/JMPR12.822

Javed, M.T., Akram, M.S., Habib, N., Tanwir, K., Ali, Q., Niazi, N. K., Gul, H., & Iqbal, N. (2018). Deciphering the growth, organic acid exudations, and ionic homeostasis of *Amaranthus viridis* L. and *Portulaca oleracea* L. under lead chloride stress. *Environmental. Science and Pollution Research*, *25(3)*, 2958-2971. https://doi.org/10.1007/s11356-017-0735-2

Khan, M., Musharaf, S., Ibrar, M., & Hussain, F. (2011). Pharmacognostic evaluation of the *Amaranthus viridis* L. *Research in Pharmaceutical Biotechnology*, *3*(1), 11-16.

Kumari, S., Elancheran, R., & Devi, R. (2018). Phytochemical screening, antioxidant, antityrosinase, and antigenotoxic potential of *Amaranthus viridis* extract. *Indian Journal of Pharmacology*, *50*(3), 130-138. http://dx.doi.org/10.4103/ijp.IJP_77_18

Mareshvaran, U.R., Murugan, L., Salvamani, S., Sharma, M., Okechukwu, P.N., & Gunasekaran, B. (2020). *In vitro* Inhibitory potential of *Amaranthus viridis* against alpha-amylase for diabetes and the antioxidant activity. *Malaysian Journal of Biochemistry & Molecular Biolology*, *2*, 127 – 134.

Naeem, Z., Jabeen, K., & Iqbal, S. (2022). Management of ochratoxigenic fungi by phytochemicals of *Amaranthus viridis* L. *Journal of Animal and Plant Sciences*, *32*(4), 1136-1142. http://doi.org/10.36899/JAPS.2022.4.0518

Olarewaju, O.A., Alashi, A.M., Taiwo, K.A., Oyedele, D., Adebooye, O.C., & Aluko, R.E. (2018). Influence of nitrogen fertilizer micro-dosing on phenolic content, antioxidant, and anticholinesterase properties of aqueous extracts of three tropical leafy vegetables. *Journal of Food Biochemistry*, 1-13. https://doi.org/10.1111/jfbc.12566

Oluwagunwa, O.A., Alashi, A.M., & Aluko, R.E. (2021). Inhibition of the *in vitro* activities of α-amylase and pancreatic lipase by aqueous extracts of *Amaranthus viridis*, *Solanum macrocarpon* and *Telfairia occidentalis* leaves. *Frontiers in Nutrition*, *8*, 1-17. https://doi.org/10.3389/fnut.2021.772903

Owuso, E., Ahorlu, M.M., Afutu, E., Akumwena, A., & Asare, G.A. (2021). Antimicrobial activity of selected medicinal plants from a Sub-Saharan African Country against bacterial pathogens from post-operative wound infections. *Medical Sciences*, *9*(2), 23. https://doi.org/10.3390/medsci9020023

Oyaizu, M. (1986). Studies on product of browning reaction - Antioxidative activities of products of browning reaction prepared from glucose amine. *The Japanese Journal of Nutrition and Dietetics, 44*(6)*,* 307–315.

Pl@ntNet. (2024, Marc 22). Amaranthus viridis L. https://identify.plantnet.org/k-world-flora/species/Amaranthus%20viridis%20L./data

Popoola, O.O. (2022). Phenolic compounds composition and in vitro antioxidant activity of Nigerian *Amaranthus viridis* seed as affected by autoclaving and germination. *Measurement: Food, 6,* 1-7. https://doi.org/10.1016/j.meafoo.2022.100028

Pulipati, S., Babu, P.S., & Narasu, M.L. (2014). Phytochemical and pharmacological potential of *Amaranthus viridis* L. *International Journal of Phytomedicine*, *6*(3), 322-326.

Reyad-ul-Ferdous, Md., Shahjahan, D. M. S., Tanvir, S., & Mukti, M. (2015). Present biological status of potential medicinal plant of *Amaranthus viridis*: A comprehensive review. *American Journal of Clinical and Experimental Medicine*, *3*(5-1), 12-17. http://dx.doi.org/10.11648/j.ajcem.s.2015030501.13

Rose, A.L., Starlin, M., Wilsy, I., & Appavoo, R. (2021). Antibacterial activity of *Amaranthus viridis* L. *International Journal of Innovative Research in Technology, 7*(11), 420-422.

Sadia, S., Mashwani, Z.U.R., Amin, H., Shedayi, A.A., Zhang, J-T., Bai, X., Nayyar, B.G., & Mazari, P. (2016). Qualitative and quantitative phytochemical analysis and antioxidant potential of *Amaranthus Viridis* L. from Pakistan. Proceedings of 54th The IIER International Conference, Beijing, China, 13th January 2016, ISBN: 978-93-82702-35-1, 10-16.

Sarker, U., & Oba, S. (2019). Nutraceuticals, antioxidant pigments, and phytochemicals in the leaves of *Amaranthus spinosus* and *Amaranthus viridis* weedy species. *Sci. Rep.*, *9:20413*, 1-10. https://doi.org/10.1038/s41598-019-50977-5

Sarker, U., & Oba, S. (2020). Phenolic profiles and antioxidant activities in selected drought-tolerant leafy vegetable amaranth. *Scientific Reports*, *10:18287*, 1-11. https://doi.org/10.1038/s41598-020-71727-y

Sarker, U., Hossain, M., & Oba, S. (2020). Nutritional and antioxidant components and antioxidant capacity in green morph Amaranthus leafy vegetable. *Scientific Reports*, *10*:1336, 1-10. https://doi.org/10.1038/s41598-020-57687-3

Sharma, S., & Vig, A.P. (2013). Evaluation of *in vitro* antioxidant properties of methanol and aqueous extracts of *Parkinsonia aculeata* L. leaves. *The Scientific World Journal*, *1*, 2–7. http://doi.org/10.1155/2013/604865

Stanković, M.S. (2011). Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts. *Kragujevac Journal of Science, 33,* 63–72.

Sunday, E.A., Gift, W.P., & Boobondah, W.J. (2021). Phytochemistry and antioxidant activity of *Amaranthus viridis* L (Green leaf). *World Journal of Advanced Research and Reviews*, *12*(02), 306–314. https://doi.org/10.30574/wjarr.2021.12.2.0468

Swarnakumari, S., Mohan, S., Sasikala, M., & Umapoorani, T. (2021). Comparative studies on *Amaranthus viridus* and *Amaranthus spinosus*. *International Journal of Pharmaceutical Sciences and Research*, *12*(10), 5618-5623. http://dx.doi.org/10.13040/IJPSR.0975-8232.12(10).5618-23

Torres, M.P., Carlos, L.A., Pedrosa, M.W., Silva, A.P.C.M., Silva, E.C., & Ferraz, L.C.L. (2018). Profile of phytochemistry and antioxidant activity of sorrel in function of organic fertilization and density of plantio. *Bioscience Journal*, *35*(3), 775-783.

Ucan Turkmen, F., & Mercimek Takci, H.A. (2018). Ultraviolet-C and ultraviolet-B lights effect on black carrot (*Daucus carota* ssp. sativus) juice. *Journal of Food Measurement and Characterization*, *12*, 1038-1046.

Zahir, S., Pal, T.K., Sengupta, A., Biswas, S., Bar, S., & Bose, S. (2021). Determination of lethal concentration fifty (LC50) of whole plant ethanolic extract of *Amaranthus Viridis*, *Cynodon Dactylon* & *Aerva Sanguinolenta* on Zebrafish (*Danio Rerio*) embryos. *International Journal of Pharmaceutical Sciences and Research*, *12*(4), 2394-2404. http://dx.doi.org/10.13040/IJPSR.0975-8232.12(4).2394-04

Zhang, Q-W., Lin, L-G., & Ye, W-C. (2018). Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese Medicine*, *13*(20), 1-26. https://doi.org/10.1186/s13020-018-0177-x

Zaware, O.R., Wakchaure, R.S., Tambe, A.V., & Lokhande, R.P. (2022). Antioxidant activity of *Amaranthus Viridis* Linn. *International Journal of Advanced Research in Science, Communication and Technology, 2*(5), 664-670. http://dx.doi.org/10.48175/IJARSCT-4880

Zengin, G., Sarikurkcu, C., Aktumsek, A., & Ceylan. R. (2014). *Sideritis galatica* Bornm.: A source of multifunctional agents for the management of oxidative damage, Alzheimer's and diabetes mellitus. *Journal of Functional Foods, 11,* 538-547. https://doi.org/10.1016/j.jff.2014.08.011

Zong, Y., Li, J., Sun, W., Liu, G., Lu, J., & Shan, G. (2016). Determination of succinic acid in desvenlafaxine succinate by high performance ion-exclusion chromatography and high performance ion-exchange chromatography. *Chinese Journal of Chromatography*, *34*(2), 189-193. https://doi.org/10.3724/SP.J.1123.2015.08017